HISTOCHEMICAL AND ULTRASTRUCTURAL STUDIES OF OOGENESIS, VITELLOGENESIS AND EGGSHELL FORMATION IN *ACANTHOPARYPHIUM SPINULOSUM* (TREMATODA: ECHINOSTOMATIDAE)

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ABSTRACT

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Histochemical and Ultrastructural Studies of Oogenesis, Vitellogenesis and Eggshell Formation in Acanthoparyphium spinulosum (Trematoda: Echinostomatidae)

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This investigation presents a composite of the cytological events of oogenesis, vitellogenesis and eggshell formation at the ultrastructural level in a poorly known echinostomid trematode, A. spinulosum. For comparison, histochemical methods which demonstrate basic proteins, phenols and phenolase were used to identify reproductive structures which produce eggshell precursors of the quinone-tanning system. It was observed that the tanning system is localized specifically in the shell globules of vitelline cells and eggshells.

Through transmission electron microscopy (TEM), it was observed that organization of the granular endoplasmic

reticulum and Golgi complex is associated with synthesis of cortical granules in dveloping oocytes. At the onset of maturation of ova, the cortical granules are characteristically arranged immediately beneath the plasma membrane. The nucleus of mature oocytes have dispersed chromatin, which suggests that oogenesis proceeds as far as the diplotene stage of the first division of meiosis. Furthermore, it was observed that "nucleolus-like bodies" occur in close association with mitochondria in these cells. However, the significance of this phenomenon is not clearly understood.

The vitelline follicles contain cells in various stages of vitellogenesis and a number of nurse cells. In contrast to immature cells, maturing vitelline cells are characterized by a well-organized granular endoplasmic reticulum and Golgi complex, which are associated with the synthesis of shell globules. These globules are arranged beneath the plasma membrane of mature cells. These cells also contain copious amounts of lipid droplets and glycogen granules. In addition, it was observed that cytoplasmic extensions of nurse cells form junctional complexes with vitelline cells, suggesting that a mechanism of selective transport exists between these cells.

There is evidence that initiation of eggshell formation occurs in the distal ootype and proximal uterus. Reticular

secretions from membranous body cells of Mehlis' gland were seen on the inner and outer surfaces of egg capsules in these regions. Granular secretions from dense body cells of Mehlis' gland were also observed adhering to egg capsules in the distal uterus. In contrast to young eggs, the older shells are several times thicker and appear to be blotched with electron-lucid areas, some of which have finely granular cores. It is suggested that the appearance of the older capsules may represent some phase of the tanning process.

In summary, egg formation in Acanthoparyphium spinulosum appears to follow the general pattern of most other trematodes. However, when comparing oogenesis and vitellogenesis of A. spinulosum to those processes in other trematodes, particularly Schistosoma mansoni and Fasciola hepatica, a greater variance was observed in the histochemistry and ultrastructure of mature vitelline cells. The degree of variance among species may be a reflection of differences in environmental factors which affect development of the eggs.

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ABBREVIATIONS

TEM ..... transmission electron microscopy

DMSO ..... dimethyl sulfoxide

MB(S1)-cell ..... membranous body cell

DB(S2)-cell ..... dense body cell

PAS ..... periodic acid-Schiff

O ..... ovary

OV ..... oviduct

OT ..... ootype

U ..... uterus

T ..... testis(es)

V ..... vitellaria

Og ..... oogonium

Oc ..... oocyte

SG ..... shell globule

ES ..... eggshell

VD ..... vitelline duct

N ..... nucleus

GER ..... granular endoplasmic reticulum

SER ..... smooth endoplasmic reticulum

G ..... Golgi complex

GV ..... Golgi-associated vesicle

M ..... mitochondrion

Ly ..... lysosome

PO ..... peripheral oocyte

CO ..... central ovum

Nu ..... nucleolus

CG ..... cortical granule

PM ..... plasma membrane

ICS ..... intercellular space

R ..... ribosome

NLB ..... nucleolus-like body

L ..... lipid droplet

MT ..... microtubule

SGC ..... cluster of shell globules

Gly ..... glycogen granules

VC ..... vitelline cell

NE ..... cytoplasmic extension of the nurse cell

DBC ..... dense body cell

OE ..... epithelium of the ootype

BL ..... basal lamina

CM ..... circular muscle

MB ..... membranous body

ci ..... cilium

Lm ..... lamellae

PU ..... proximal uterus

S1 ..... secretory product of the membranous body cell

DBP ..... process of the dense body cell

CHAPTER I

INTRODUCTION

Acanthoparyphiunm spinulosum is a trematode which inhabits the small intestine of the Australian golden plover, Charadrius dominicus, and other shore birds, such as Recurvirostra americana and Squatarola squatarola, in Japan and California. This digenetic trematode requires a brackish-water snail as the first intermediate host. The life cycle of A. spinulosum, initially described by Bearup (1960) in Australia, has been experimentally worked-out by Martin and Adams (1961), using the domestic chick which had been fed infected radular muscle. The eggs of the adult worm pass in feces from birds and hatch into miracidia which infect the snail, Cerethidea californica. Two successive generations of rediae develop within the digestive gland of the snail. Subsequently, motile cercariae exit the snail host and, after swimming freely for a short time, reenter C. californica and encyst in the radular muscle as metacercariae. In birds that feed on the infected snails, the metacercariae excyst in the small intestine and develop into adults within 12 days. In this cycle, C. californica serves as

both the first and second intermediate hosts. However, there is evidence that the commercial oyster, Crassostrea virginica, along the southern coast of Texas is naturally infected with the metacercaria of Acanthoparyphium spinulosum. The larva can excyst and survive in rats (Little et al., 1966).

Crassostrea virginica may serve as a potential vector for infections of A. spinulosum in humans, considering that adult trematodes are harbored in virtually every class of vertebrates and that human infections may be limited only by infrequent ingestion of uncooked or poorly cooked intermediate hosts. Since the diagnosis of fluke infections, such as schistosomiasis, frequently depends on specific identification of eggs, an investigation of egg formation in A. spinulosum is warranted.

Based on studies of digenetic trematodes of widely separated taxa, gametogenesis and eggshell formation appear to follow a general pattern, with a number of minor variations. Of the taxonomic groups that have been investigated, Fasciola hepatica has received the greatest attention (Kouri and Naus, 1938; Yosufzai, 1953; Hanumantha-Rao; Gonnert, 1962; Bjorkman and

Thorsell, 1963; Irwin and Threadgold, 1972; Threadgold, 1982). Except for the light microscopic study by Shropshire (1984), there is no information available on the cytological details of gametogenesis and eggshell formation in A. spinulosum. This investigation is designed to study the ultrastructure of egg formation in A. spinulosum, with emphasis on the ovary, vitelline follicle and encapsulated egg. For comparison, histochemical methods were used to identify female genitalia that produce eggshell precursors. Since both Fasciola hepatica and A. spinulosum are members of the order Echinostomatida, ultrastructural studies of egg formation in species other than F. hepatica may reveal greater diversity or similarity among related trematodes.

CHAPTER II

REVIEW OF LITERATURE

The Maturation of Oocytes

Goldschmidt (1908) was the first to describe oogenesis in trematodes, using Dicrocoelium dendriticum (D. lanceolatum). Since then, Gresson (1964a) has reviewed a number of studies which describe oogenesis in various trematodes. The ultrastructural aspects of oogenesis have been reported for several families, including Fasciolidae, Gorgoderidae, Troglotrematidae, Plagiorchiidae, Schistosomatidae, Diplostomatidae, Aspidogastridae and Didymozoidae (Bjorkman and Thorsell, 1964; Koulish, 1965; Sato et al., 1966; Burton, 1967a; Spence and Silk, 1971; Grant et al., 1977; Hathaway, 1979; Justine and Mattei, 1984).

Although minor variations among taxonomic groups have been reported, the principal cytological features of oogenesis in trematodes are similar (Gresson, 1964a). In general, oogenesis proceeds as far as the first meiotic prophase in the primary oocyte. The process typically begins at the periphery of the ovary, which contains many small oogonia and early, immature oocytes. Both light and electron microscopic studies of

these cells indicate that they are indistinguishable or barely indistinguishable in appearance (Burton, 1960; Koulish, 1965; Halton et al., 1976). The most prominent feature of these cells is a large nucleus which occupies most of the cell volume. The nucleus contains dense masses of chromatin and, at least, one prominent nucleolus. Numerous free ribosomes are scattered throughout the cytoplasm. The endoplasmic reticulum, when present, is very sparse with relatively few ribosomes attached. Golgi complexes and mitochondria are few in number. The mitochondria are generally found in small groups in close proximity to the nucleus at one or both poles.

Developing oocytes are more centrally located within the ovary and are characterized by an increase in size along with the appearance of cortical granules. However, such granules seem to be absent in the fish blood fluke, Aporocotyle simplex (Thulin, 1982). The granular endoplasmic reticulum and Golgi complex become organized extensively in association with the synthesis of cortical granules, which increase in number and become randomly distributed in the cytoplasm. The granules are spherical when fully formed and vary in

size and content from species to species. In Schistosoma mansoni, these bodies are approximately 1 μm in diameter and consist of several concentric lamellae with a core of granular material (Erasmus, 1973). Grant et al. (1977) have reported smaller cortical granules, approximately 0.18 μm, in Pharyngostomoides procyonis. According to Halton et al. (1976), similar ranges also exist in monogenetic trematodes. The granules have been described as containing either several dense, core-like structures in a fairly fibrous matrix or coreless ones with dense fibrous or granular material.

Other features of developing oocytes include an increase in the number of mitochondria, except in Haematoloechus medioplexus (Burton, 1960), and nuclear changes. Yosufzai (1953) and Gresson (1958) reported that mitochondria become redistributed around nuclei of oocytes in Fasciola hepatica and Sphaerostoma bramae, respectively. Burton (1960) studied maturing oocytes of H. medioplexus, histochemically, and observed large nuclei in the diplotene stage. Further evidence that early prophase of the first meiotic division proceeds as far as the diplotene stage in developing oocytes has been reported in S. bramae by Gresson (1958), in

Neorenifer wardi by Dunn (1959) and in Schistosoma mansoni by Erasmus (1973). Halton et al. (1976) reported that most of the chromatin is dispersed as euchromatin in developing oocytes of the monogenetic trematodes, Diclidophora merlangi, Diplozoon paradoxum and Calicotyle kroyeri. Nuclear extrusions have been observed in the perinuclear regions of oocytes in a number of digenetic trematodes (Gresson, 1964a) and other organisms, such as crustaceans (Beams and Kessel, 1963; 1980), sea squirts (Kessel, 1966), nematodes (Adamson, 1982) and spider mites (Wagner and Seitz, 1984). Yosufzai (1953) first postulated that the nuclear material was of nucleolar origin in Fasciola hepatica. Gresson (1964b) demonstrated, ultrastructurally, that groups of granules passed from the nucleolus into the nucleoplasm in oocytes of F. hepatica and suggested the feasibility of nucleolar material migrating to the cytoplasm. This observation was in accord with those of Bjorkman and Thorsell (1964) which revealed "ribosomal bodies" in the cytoplasm of F. hepatica oocytes. Koulish and Kleinfeld (1954) demonstrated, histochemically, that a large, dense structure in the cytoplasm of developing oocytes in Gorgoderina

attenuata contained ribonucleoprotein. Koulish (1965) described the "nucleolus-like" body in G. attenuata oocytes as an irregularly shaped mass of densely packed, ribosome-like particles (150-200 A in diameter) unbound by a membrane. Similar ultrastructural details of this nucleolus-like body have been described in Haematoloechus medioplexus by Burton (1967a), in Pharyngostomoides procyonis by Grant et al. (1977) and Gonapodasmius by Justine and Mattei (1984). Further, Halton et al. (1976) observed that small aggregates of dense material occur on both sides of the nuclear pores and that the pores contain a core of granular material. Such findings led these investigators to suggest that passage of RNA to the cytoplasm may be associated with nuclear extrusions in maturing oocytes of monogenetic trematodes.

When compared to developing oocytes, the mature oocytes are larger and located at the central and posterior portions of the ovary near the oviduct. The nucleus contains dispersed euchromatin and a well-developed nucleolus. The numerous mitochondria and free ribosomes are distributed throughout the cytoplasm. The most conspicuous characteristic of mature oocytes is the

regular arrangement of the cortical granules immediately beneath the plasma membrane (Bjorkman and Thorsell, 1964; Erasmus, 1973; Halton et al., 1976; Grant et al., 1977; Hathaway, 1979; Justine and Mattei, 1984). A similar arrangement of cortical granules also exists in mature oocytes of other flatworms, including cestodes (Mokhtar-Maamouri, 1980) and turbellarians (Boyer, 1972; Gremigni and Domenici, 1975; Gremigni and Nigro, 1983). The peripheral position of the cortical granules persists until after the primary oocyte reaches the specialized region of the oviduct, the ootype.

Halton et al. (1976) reported that cortical granules appeared to be released from the oocytes of Diclidophora merlangi following fertilization, even though the exact course of events had not been defined. Similarly, Justine and Mattei (1984) reported that the fertilized oocytes of Gonapodasmius were surrounded by numerous electron-dense granules which originated, in part, from cortical granules of the virgin oocytes. The observation that cortical granules are involved in the initial phase of fertilization has also been reported in echinoderms by Anderson (1968) and Holland (1979), in a polyclad turbellarian by Boyer (1972) and in nematodes

by Adamson (1982) and Preston and Jenkins (1984).

According to Holland (1979), the cortical granules which are released from fertilized oocytes of the echinoderm, Ophiopholis aculeata, adhere to a vitelline coat to form a fertilization envelope. Similarly, Adamson (1982) reported that cortical granules are involved in the initiation of eggshell formation in the nematode, Gyrinicola batrachiensis.

As oocytes mature, they begin to separate, become more oval and are released into the oviduct. They are surrounded by spermatozoa and vitelline cells, which contain yolk material and shell precursors, as they pass through the oviduct to the ootype. Vitelline cells are produced in separate follicles, the vitellaria, which shed their cells into ducts leading to the oviduct (Willey and Koulish, 1950; Smyth, 1951; Burton, 1963; Nollen et al., 1976; Rees, 1979).

The Maturation of Vitelline Cells

As in oocyte development, the vitelline follicles contain cells in various stages of maturation. There is, however, a wider range of variation in the histochemical and ultrastructural features of vitellogenesis

from species to species among trematoes. Ultrastructural studies, which describe in some detail the development of vitellaria, have been limited to a few species. These studies include those on Fasciola hepatica by Irwin and Threadgold (1970) and Threadgold (1982), on Schistosoma mansoni by Erasmus (1973; 1975a), on Gorgoderina vitelliloba by Irwin and Maguire (1979) and on Pharyngostomoides procyonis by Grant et. al. (1977).

Irwin and Threadgold (1970) and Irwin and Maguire (1979) observed immature cells and a number of nurse cells at the periphery of the follicle. Immature cells have a large, central nucleus which contains dense areas of chromatin and often a nucleolus. There are numerous free ribosomes and, in most cases, mitochondria throughout the cytoplasm. Grant et al. (1977) reported that mitochondria are sparse at this stage in P. procyonis. The granular endoplasmic reticulum and Golgi complex are sparse or absent. According to Irwin and Threadgold (1970), the nurse cells of F. hepatica have an irregularly shaped nucleus in which a nucleolus is rarely found. The cytoplasm contains large mitochondria, a conspicuous smooth endoplasmic reticulum and Golgi

complex, microtubules and glycogen granules. These cells have cytoplasmic extensions which branch between and around the vitelline cells.Junctional complexes have been observed both between parenchyma and developing vitelline cells with nurse cells in Fasciola hepatica by Irwin and Threadgold (1970) and in Gorgoderina vitelliloba by Irwin and Maguire (1979).

As reported by Irwin and Threadgold (1970), vitelline cells enlarge and migrate to the center of the follicle during maturation. These cells commonly have a number of cytoplasmic inclusions not found in immature cells. The granular endoplasmic reticulum is extensive and consists of long strands in parallel array. The Golgi complex is associated with small, electron-dense vesicles, the shell globules. As maturation continues, whorls of the granular endoplasmic reticulum become scattered throughout the cytoplasm. The Golgi complex becomes sparse and the shell globules coalesce to form large, membrane-bound clusters, which migrate to the periphery of the cell.

As reviewed by Frayha and Smyth (1983), copious amounts of lipid droplets have been detected in vitelline cells of many species of digenetic trematodes. In

Schistosoma mansoni, mature cells are rich in lipid droplets, but have very little glycogen, according to Erasmus (1973). In contrast, Irwin and Threadgold (1970) observed high concentrations of glycogen and little or no lipid droplets in mature cells of F. hepatica. Halton et al. (1974) detected moderate to high concentrations of glycogen and lipid droplets in mature vitelline cells of monogenetic trematodes. Furthermore, these investigators observed that the glycogen content differed between species of Didlidophora, which they attributed to a difference in the length of time the eggs are retained in the uterus of these species.

The Tanning of Eggshells

Histochemical and autoradiographic studies of the chemical composition of shell globules have led to the identification of the shell material as precursors of scleroprotein in many digenetic trematodes (Smyth and Clegg, 1959; Guilford, 1961; Burton, 1963; Ma, 1963; Nollen et al., 1973; Srivastava and Gupta, 1978; Seed and Bennett, 1980). These precursors have been identified as basic and tyrosine-rich proteins, phenols and phenolase, which constitute a quinone-tanning

system. Phenolase is considered to be a complex enzyme or combination of enzymes capable of attacking a wide range of mono- and diphenols (Smyth and Clegg, 1959). The terms polyphenol oxidase and tyrosinase have been used to describe this complex. Evidence indicates that ortho-quinone is derived enzymatically from the oxidation of phenolic compounds. According to Smyth (1954) and Johri and Smyth (1956), the basic reactions in quinone-tanning systems in trematodes and pseudophyllidean cestodes may be summarized as follows:

protein+diphenol $\xrightarrow{\textbf{polyphenol oxidase}}$ protein+quinone

= tanned protein (sclerotin)

Quinone reacts with free amino or sulfhydryl groups on adjacent protein chains to form cross-linked and hardened protein. The subsequent conversion of quinone to melanin causes the protein to tan. Burton (1963) showed that the vitelline cells and young eggshells of Haematoloechus medioplexus contain tyrosine-rich proteins and dihydroxyphenylalanine oxidase.

Although the stabilization of eggshell protein may occur via the process of quinone-tanning in many digenetic trematodes, it has been recognized that a number of species contain precursors for tanning, yet

have colorlessand transparent shells. In a comparative study of three species of digeneans with such shells, Philophthalmus megalurus, Gorgoderina attenuata and Megalodiscus temperatus, Nollen (1971) demonstrated that the vitelline cells of all three species gave positive reactions for basic protein and tyrosine. However, phenolase was detected only in those cells of P. megalrus, using either catechol or dihydroxyphenylalanine as substrate. Nollen (1971) suggested that the lack of tanning in the eggshell of P. megalurus was due to the presence of a partial enzyme system in which tyrosinase was absent, thereby, preventing the hydroxylation of tyrosine to a dihydroxyphenol and blocking the production of quinone. Srivastava and Gupta (1976) showed that, even though the mature eggs of Isoparorchis hypselobagri are also colorless and transparent, the shell globules of vitelline cells and the immature eggs were strongly positive for basic proteins, phenols and phenolase. They also observed that the mature eggs lost their ability to stain for phenols, which they attributed to the conversion of phenols to quinones. Srivastava and Gupta (1978) demonstrated, colorimetrically, the presence of a quinone-tanning system in I. hypselobagri

by showing that the eggs were positive for polyphenol oxidase and sclerotin/melanin.

The Role of Mehlis' Gland in Eggshell Formation

Investigators originally thought that the eggshell precursors were secreted by Mehlis' gland, which surrounds and empties into the ootype. Burton (1976b) described two types of secretory cells as membranous body (MB)-cells and dense body (DB)-cells in Haematoloechus medioplexus. Threadgold and Irwin (1970) described these cells in Fasciola hepatica as S1- and S2-cells, respectively. The MB(S1)-cells deposit a thin membrane around the egg and associated vitelline cells. It is believed that this membrane may cause shell globules to release their contents (Hanumantha-Rao, 1959; 1960; Clegg, 1965; Irwin and Threadgold, 1972). Hanumantha-Rao (1959) and Burton (1963) reported that secretions from MB(S1)-cells contained phospholipid. However, Clegg (1965) suggested that these cells secrete lipoprotein and showed that these membranes adhere to the inner and outer surfaces of eggshells in Fasciola hepatica. Clegg and Morgan (1966) used thin layer chromatography to compare the lipid composition of the lipoprotein in these membranes and eggshells. They

demonstrated that the two sources contained closely similar phospholipids and a glycolipid that were absent from the posterior end of F. hepatica. Moreover, Bogitsh (1970) reported that DB(S2)-cells of Haematoloechus medioplexus, in contrast to MB(S1)-cells, reacted positively for macromolecular diglycols. These results are in accord with the histochemical findings reported by Irwin and Threadgold (1972), which indicate that S2 secretions in F. hepatica are acid mucopolysaccharides.

Based on the first direct evidence that phenolase existed as a proenzyme in monogeneans, Pricea and Protomicrocotyle, Ramalingam (1970) suggested that Mehlis' gland may play a role in the activation of prophenolase. He observed that vitelline cells in live and briefly fixed specimens were nonreactive to catechol; whereas, those of cells in longer fixed specimens were reactive. He also observed that sodium oleate treatment activated prophenolase in the helminth system. More recently, the existence of prophenolase and its activation in trematodes has been demonstrated in Schistosoma mansoni by Seed et al. (1978), in Paraplerurus sauridae by Nellaiappan and Ramalingam

(1980a; 1980b) and in Fasciola hepatica by Thangaraj et al. (1982). Based on the report by Bogitsh (1970) in Haematoloechus that indicated the presence of acid phosphatase in Mehlis' gland, Nellaiappan and Ramalingam (1980b) suggested that secretions from Mehlis' gland may cause limited lysis of prophenolase. Thangaraj et al. (1982) used extracts of Mehlis' gland and artificial activators and found that the activation power of extracts was only half that of sodium oleate. In addition, electrophoretic analysis of prophenolase led these investigators to suggest that activation of the proenzyme results in its dissociation into activated subunits, which is in accord with observations of Nellaiappan and Ramalingam (1980b).

CHAPTER III

MATERIALS AND METHODS

Source of Acanthoparyphium spinulosum

Cerethidea californica snails, obtained from Pacific Bio-Marine Laboratories, Venice, CA and Jones Biomedical Laboratories, Long Beach, CA, were maintained in brackish water in fingerbowls at room temperature. Infected snails were isolated when motile cercariae were observed in the sea water. When identified as A. spinulosum, cercariae were used to reinfect isolated snails. Radular tissues infected with metacercariae were removed from snails and fed to day-old hatchery-raised chicks. Infected chicks were maintained under fasting conditions for 24 hr post-infection. Adult worms were removed from the lower duodenum and upper ileum in physiological saline (0.85%) at necrospy 9-14 days after infection.

Histochemical Methods

Histochemistry was used to demonstrate eggshell material in the reproductive system. The following methods of Johri and Smyth (1956) were used:

1. Bromophenol Blue Method

Bromophenol blue was used to demonstrate basic proteins. Sections were washed in distilled water and overstained in 0.1% aqueous bromophenol blue. They were washed in 0.5% acetic acid for 20 min and 3 min in tap water. Afterwards, sections were dehydrated through a graded series of ethanol (50%, 70%, 95%). If sections remained overstained at this point, they were differentiated in absolute ethanol (100%), cleared in xylene and mounted in Permount.

2. Malachite Green Method

Malachite green was also used to demonstrate basic proteins. Sections were washed in distilled water and stained in Gower's carmine for 12 hr. They were rinsed in distilled water and overstained in 0.5% aqueous malachite green. Sections were rinsed in distilled water and counterstained in 1% orange G in 100% ethanol for 5 sec. Afterwards, they were rinsed in 100% ethanol, cleared in xylene and mounted in Permount.

3. Catechol (Ortho-dihydroxybenzene)

Catechol was used as a substrate to demonstrate phenolase activity. Sections were washed in distilled water and stained in 0.1% fresh catechol for 60-90 min

at 40 C. They were washed for 15 min in distilled water, then stained in Gower's carmine for 12 hr. Sections were differentiated in 0.5% HCl in 70% alcohol for 3 hr, dehydrated, cleared and mounted in Permount. Control specimens were pretreated with 0.001 M potassium cyanide (Smyth, 1954) to inactivate phenolase.

4. Fast Red Salt B

Fast red salt B was used to demonstrate phenolic groups. This method was suitable in cases where phenolase may have been destroyed during fixation. Sections were washed in distilled water and stained in freshly filtered solutions of 1% aqueous fast red salt B for 40 min. They were washed in distilled water for 15 min, dehydrated, cleared and mounted in Permount.

For comparison, the complete morphology of sectioned material was demonstrated, using the method of Halton (Department of Zoology, Queen's University, Belfast, Ireland: personal communication) as follows:

Sections were washed in distilled water, stained in 0.5% aqueous acid fuchsin for 5 min., washed again in distilled water and blotted dry. Afterwards, they were stained in 0.5% aqueous toluidine blue for 3 min, washed and blotted. Sections were dehydrated, cleared and

mounted in Permount.

Cryostat Method for Preparation of Specimens

The cryostat was used to obtain longitudinal sections (8-10 µm) from whole specimens mounted in O.T.C. embedding medium. Specimens were frozen to -20 C and sectioned in an Ames II cryostat. Sections were collected on coverslips. Then, coverslips were warmed to allow the tissue to adhere to them. Afterwards, tissues were placed, facing upward, in a petri dish, fixed in 70% alcohol and stained according to the desired histochemical method.

Ultramicrotome Method for Preparation of Specimens

The ultramicrotome was used to obtain both longitudinal and cross sections (1-3 µm) from whole specimens, using the method of Halton (personal communication). The specimens were fixed in 3% glutaraldehyde in 100 mM Millonig phosphate buffer (pH 7.2) with 1.5% sucrose for 3 hr at 4 C. Following fixation, specimens were thoroughly washed at 4 C in 100 mM Millonig buffer (pH 7.2) with 5% sucrose and dehydrated through a graded series of ethanol (50% , 70%, 95%). The dehydrated samples were infiltrated for at least 3 hr at 25 C in fresh JB-4 catalyst solution and polymerized at 25 C for

JB-4 embedding solution. The specimens were sectioned with glass knives on an LKB III ultramicrotome and collected on clean glass slides. They were, then, flattened in 1% ammonium hydroxide, dried at 45 C and stored overnight at 25 C before the staining procedure. Stained preparations were observed, double-blind, with a Leitz Wetzlar light microscope equipped with Leica optics.

Transmission Electron Microscopy

Fixation

Ultrastructural studies of the female reproductive system were performed using samples from whole specimens and segments fixed overnight at 4 C in one of three fixatives: (1.) 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose; (2.) a mixture of 1% glutaraldehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, total osmolality of 176 mOsm), according to McDowell and Trump (1976); and (3.) a mixture of 5% glutaraldehyde, 4% paraformaldehyde and 360 mM dimethyl sulfoxide (DMSO) in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose, as described by Robinson and Halton (1982). The mixtures of fixatives (2. and 3.), which combine two or more qualities of good fixation,

were used as controls for interpretable and undesirable artifacts.

Embedding

Before embedding tissues, segments with female genitalia were prepared from whole worms, pre- or post-fixed in one of the methods given above, in the following manner:

Specimens were slightly flattened by applying coverslips to them in a drop of the appropriate fixative on glass slides. The region posterior to the ventral sucker and anterior to the bilobed testes was identified with a dissecting microscope. The ovary, uterus and vitellaria were located in this region. Segments of this region were excised with acetone-cleaned razor blades and segments were transferred to vials with the appropriate fixative-free buffer at 4 C for 4 hr. Tissues were dehydrated through a graded series of ethanol (50%, 70%, 80%, 95%, 100%) to propylene oxide at 4 C. Tissues were then infiltrated overnight in a mixture of propylene/Spurr's low viscosity resin; after which, they were embedded in fresh Spurr's resin in closed Beem capsules and polymerized at 60 C for 48 hr.

Sectioning and Staining

Thick sections (1-3 μm) were prepared, stained with acid fuchsin/toluidine blue and used to select, light microscopically, areas for thin sectioning.

Thin sections were prepared with glass knives on the LKB III ultramicrotome and collected on 300 mesh copper grids, according to the method of Racker (1983). Sections were double-stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963). Specimens were examined with a JEOL/JEM-100CX-II electron microscope. Ultrastructural analyses in this investigation routinely required only low to mid-range (1,100 to 35,000 x) magnification.

CHAPTER IV

EXPERIMENTAL RESULTS

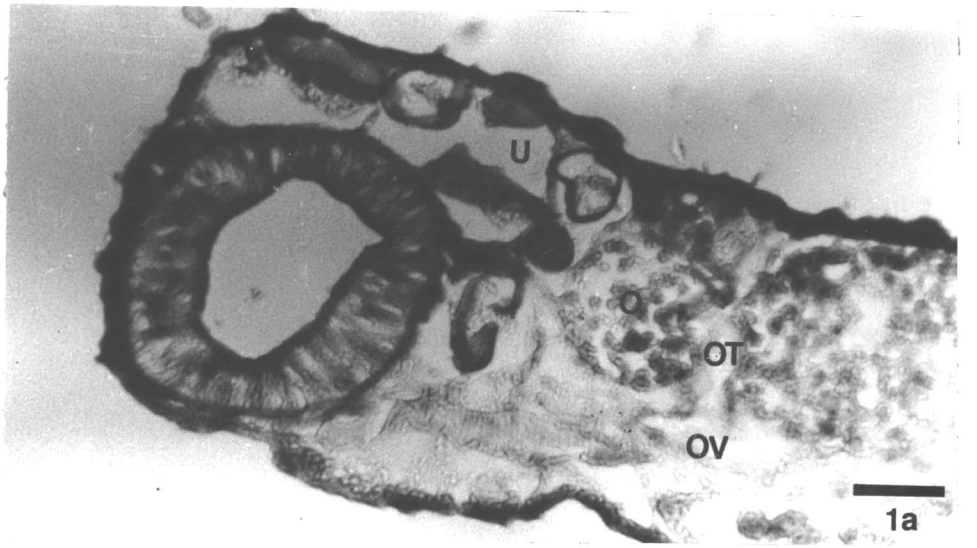
Demonstration of General Morphology of the Reproductive System

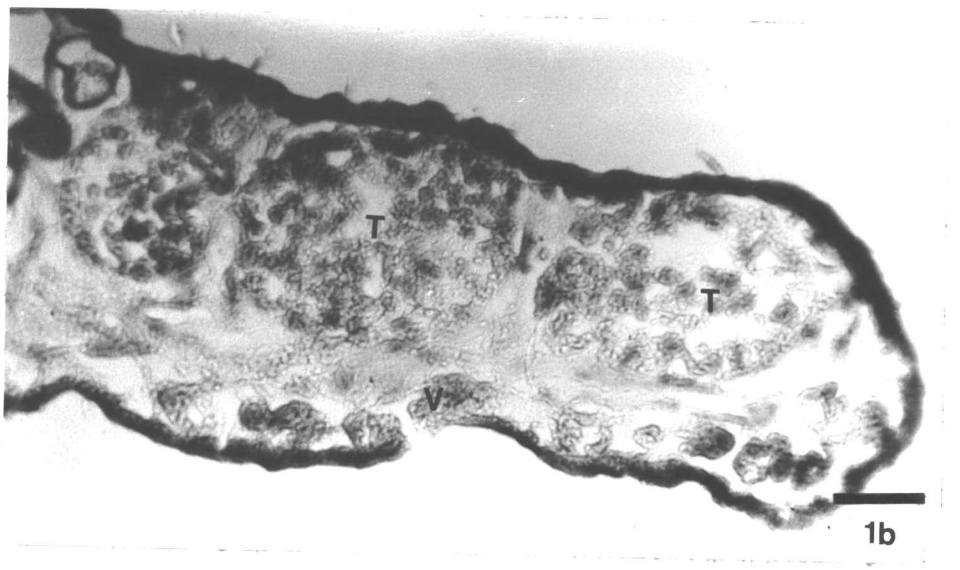
The ovary of Acanthoparyphium spinulosum is located directly anterior to tandemly paired testes and slightly to the right of the mid-saggital plane. Ducts extendanteriorly from the testes to the cirrus sac, which proceeds posterior to the acetabulum nearly to the ovary. The oviduct emerges from the posterior surface of the ovary and is joined by Laurer's canal and the main vitelline ducts before entering the ootype. This specialized structure is positioned between the ovary and the anterior testis and is encircled by Mehlis' gland cells. The vitelline follicles extend laterally from the level of the ovary to near the ends of the ceca. The uterus continues anteriorly from the ootype to the genital pore and is convoluted on each side of the body between the ootype and acetabulum.

Figures 1a and 1b show the general arrangement of the reproductive system before destaining in the bromophenol blue method. The ovary, oviduct, ootype and uterus are located in the anterior half of the organism.

Fig. 1a. Longitudinal section of the anterior half of Acanthoparyphium spinulosum, stained with bromophenol blue (before destaining). O, ovary; Ov, oviduct; OT, ootype; U, uterus. Scale bar = 30 μm.

Fig. 1b. Longitudinal section of the posterior half of Acanthoparyphium spinulosum, stained with bromophenol blue (before destaining). T, testes; V, vitellaria. Scale bar = 30 μm.


U
O
OT
OV
1a


T
T
V
1b

The vitelline follicles and tandemly paired testes are located in the posterior half of the body. In sections stained with acid fuchsin/toluidine blue, the oogonia/ immature oocytes, eggs and shell globules of vitelline cells were strongly basophilic (Figs. 2 and 3).

Histochemical Demonstration of Basic Proteins

After specimens were destained according to the bromophenol blue method, only eggshells and vitelline cells were positive for basic proteins (Table 1). A stronger reaction with bromophenol blue occurred in the eggshell than in the vitelline follicle (Fig. 4).

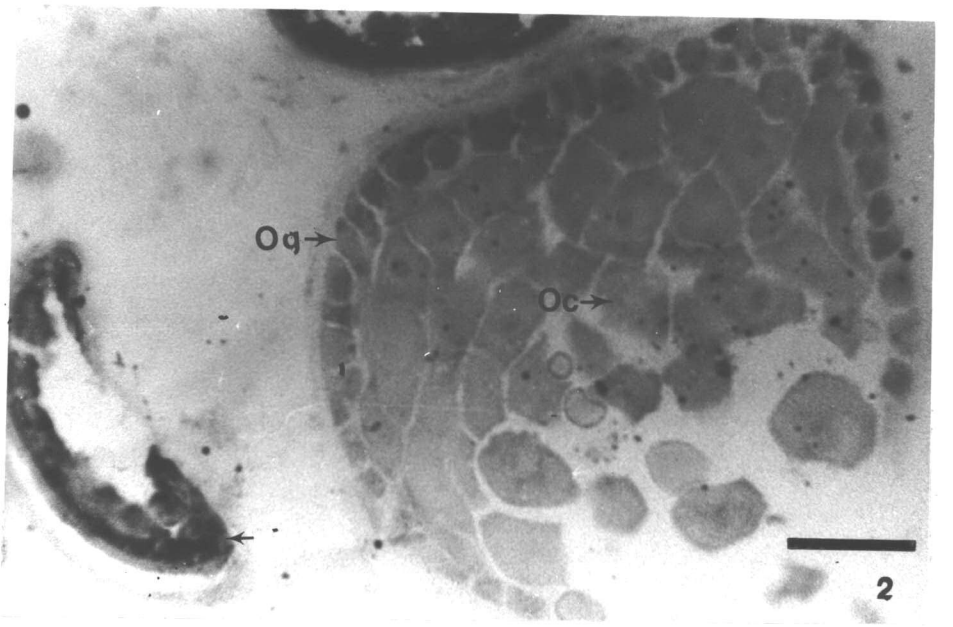
Intense malachite green stain was observed in eggshells and vitelline follicles (Table 1), where it was localized within the shell globules. This pattern was evident within the follicles and vitelline ducts (Fig.5). In contrast to eggshells and vitellaria, the ovary and testes were negative for this procedure (Fig. 6).

Histochemical Demonstration of Phenolic Compounds

When the fast red salt B method was used, only the vitellaria and encapsulated eggs were positive for phenols (Table 1). In contrast to vitelline cells,

Fig. 2. Cross section of the ovary and a portion of the uterus, stained with acid fuchsin/ toluidine blue. Og, oogonium; Oc oocyte. The arrow indicates an egg in the uterus. Scale bar = 10 μm.

Fig. 3. Cross section of a vitelline follicle, stained with acid fuchsin/toluidine blue. Note the shell globule (SG) in a mature vitelline cell. Scale bar = 5 μm.


Oo
Oc
2

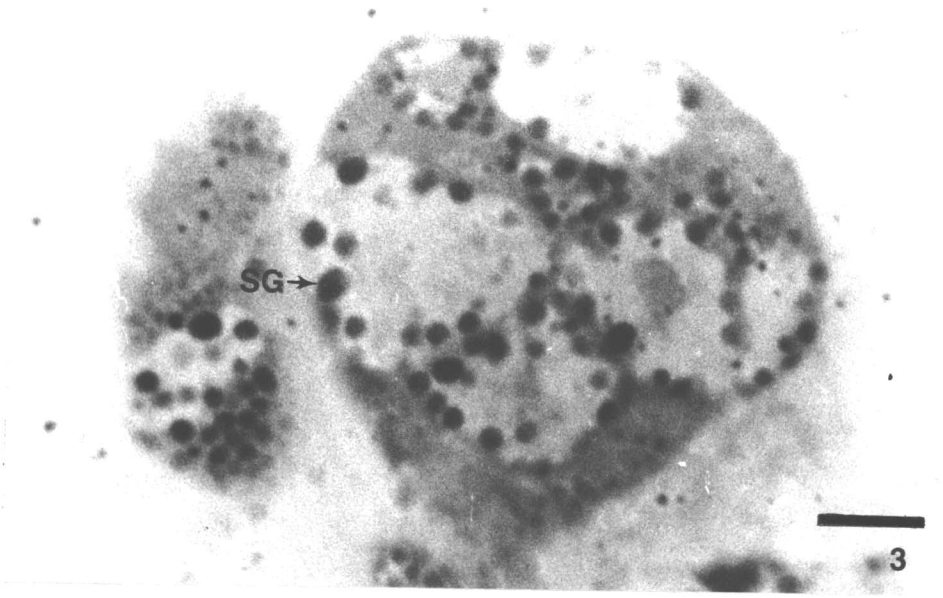

SG
3

Table 1. The localization of eggshell precursors in *Acanthoparyphium spinulosum* adults.

Reacting Substance	Test	Ovary	Testis	Vitel-laria	Egg-Shell
Proteins	Bromophenol blue	0	0	+	++
Proteins	Malachite Green	0	0	++	++
Phenols	Fast Red Salt B	0	0	++	++
Phenolase	Catechol	0	0	++	++

Key to stain reactions: 0, no stain; +, slightly stained; ++, moderately to intensely stained.

Fig. 4. Longitudinal section illustrating the egg-shell (ES) and vitellaria (V), stained with bromophenol blue (after destaining). Scale bar = 30 µm.

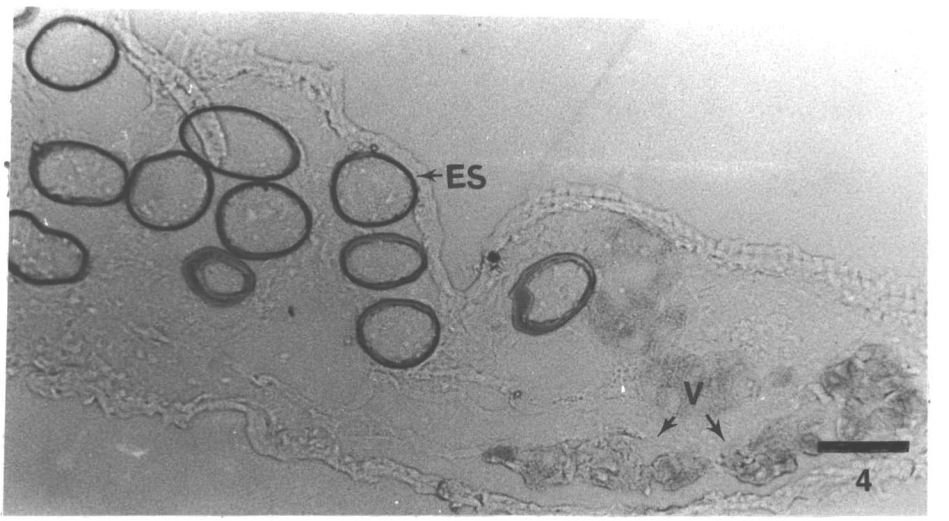
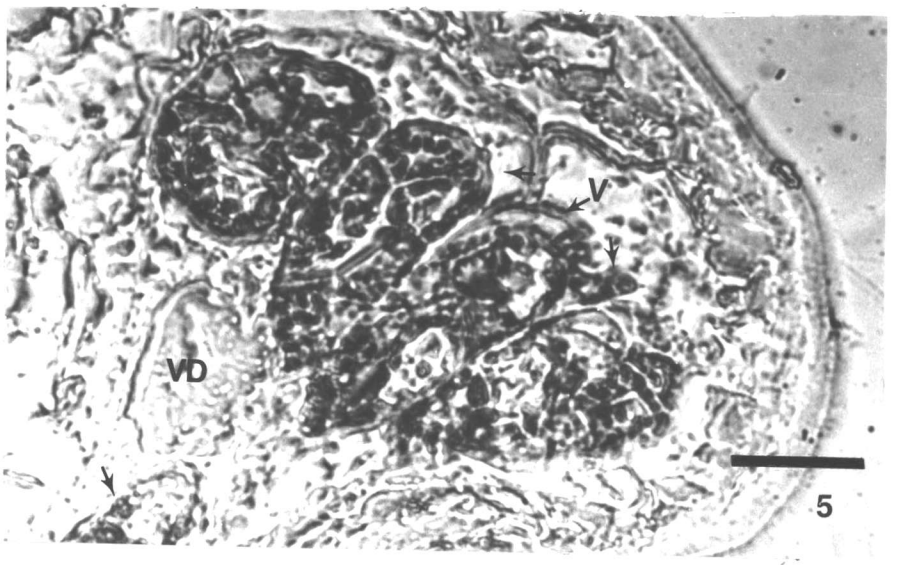
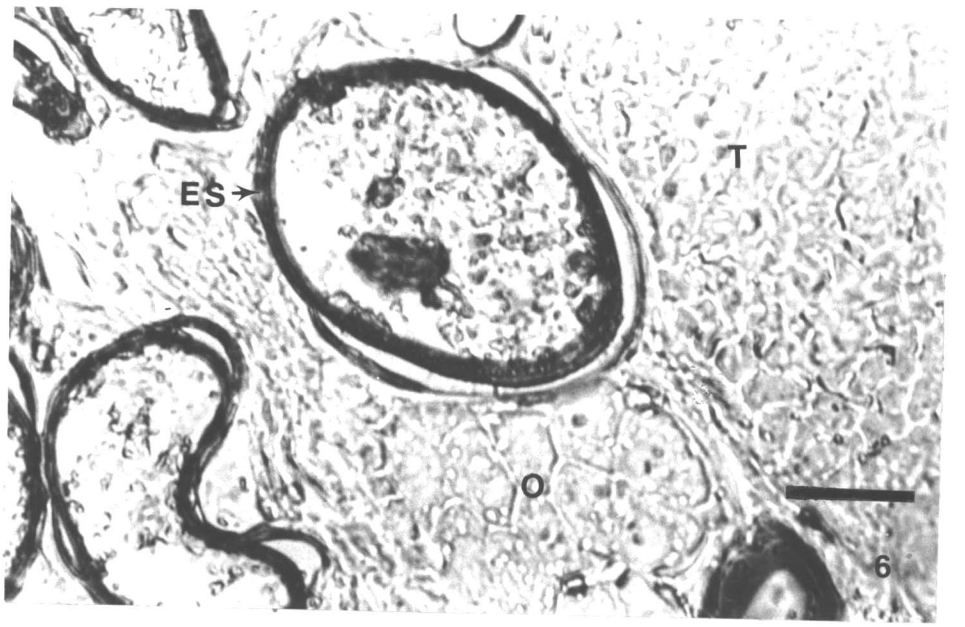

ES
V
4

Fig. 5. Longitudinal section of the vitellaria (V), stained with malachite green. The arrow indicates a mature vitelline cell in the vitelline duct (VD). Scale bar = 10 μm.

Fig. 6. Mid-region of an adult, stained with malachite green. O, ovary; T, testis; ES, eggshell. Scale bar = 10 μm.


V
VD
5


ES
T
O
6

eggshells stained less intensely (Figs. 7a and 7b). In many cases, little or no staining occurred in egg capsules in the distal uterus.

Histochemical Demonstration of Polyphenol Oxidase

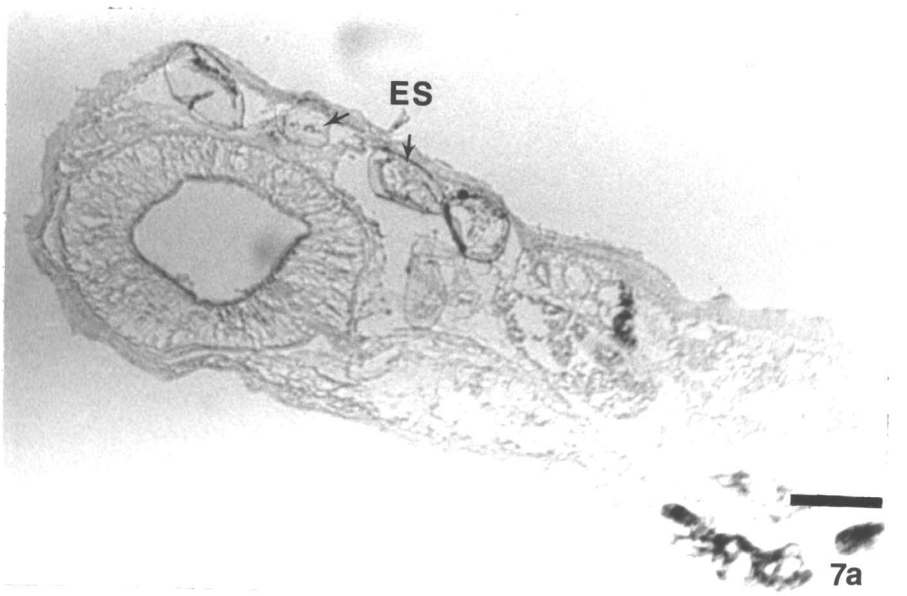
The catechol method for phenolase activity gave moderate to strong reactions in eggshells and vitellaria (Table 1). The reddish brown color, indicating phenolase activity, was evident in eggshells in the proximal uterus. Neighboring structures, including the oviduct and testes, did not stain (Fig. 8). In the case of vitelline cells, enzyme activity was associated with shell globules (Fig. 9).

Ultrastructure of the Ovary

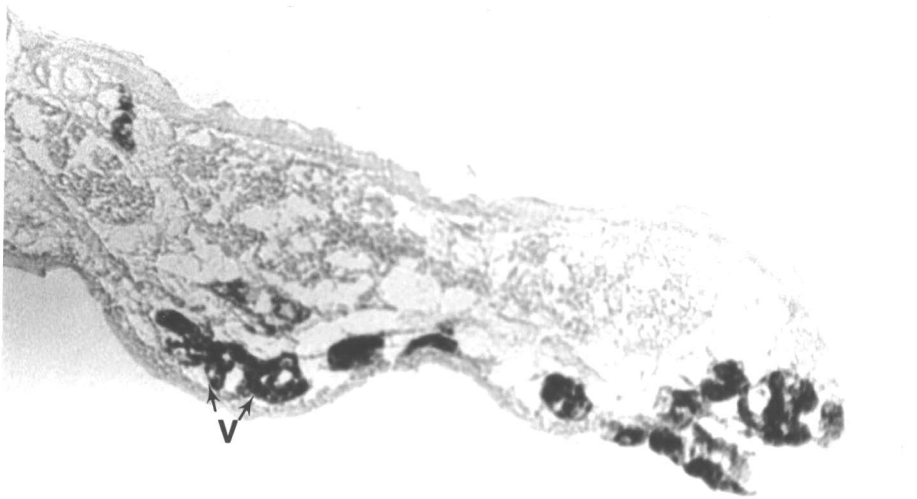
The ovary contains germ cells in various stages of development. The small oogonia and young oocytes are closely packed at the anterior end of the ovary. As seen in Fig. 10, oogonia contain large nuclei which are surrounded by thin rims of cytoplasm that appear to have numerous free ribosomes and increased numbers of mitochondria. The cytoplasm also contains a granular endoplasmic reticulum, Golgi complex and numerous aggregations of vesicles, some of which are empty or

Fig. 7a. Longitudinal section of the anterior half of an adult, stained with fast red salt B. Note the eggshell (ES) in the uterus. Scale bar = 30 μm.

Fig. 7b. Longitudinal section of the region posterior to that shown in Fig. 7a, stained with fast red salt B. Note the vitellaria along the lateral wall. Scale bar = 30 μm.



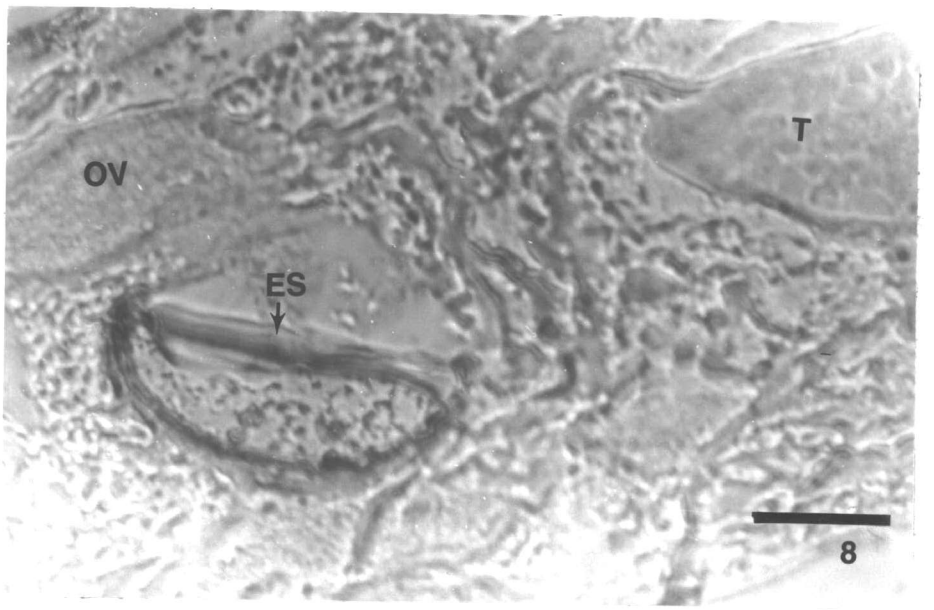
7a



7b

Fig. 8. Mid-region of an adult, stained with catechol. T, testis; OV, oviduct; ES, eggshell. Scale bar = 10 μm.

Fig. 9. Section of the vitellaria, stained with catechol. Note the shell globules (SG). Scale bar = 10 μm.


OV
T
ES
8

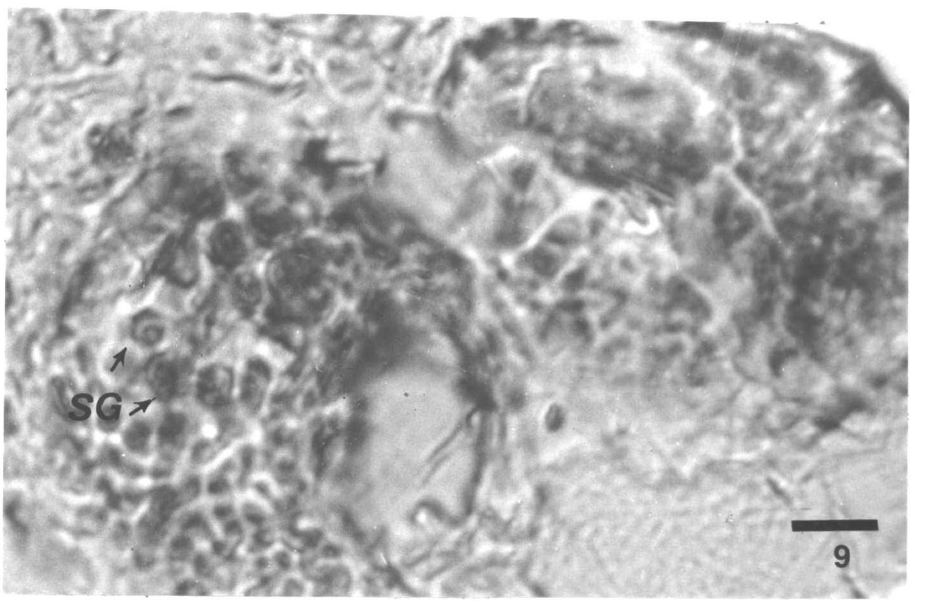
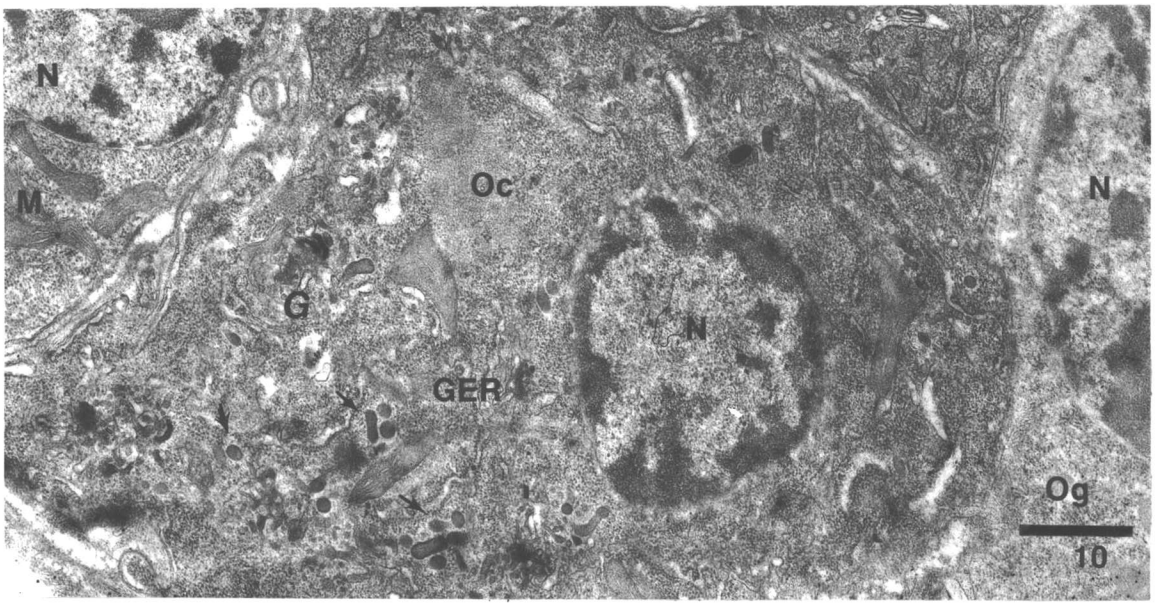

SG
9

Fig. 10. Periphery of the anterior region of the ovary illustrating an oogonium (Og) and oocyte (Oc). N, nucleus, GER, granular endoplasmic reticulum; G, Golgi complex; M, mitochondria. Arrows indicate vesicles. Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.97 μm.


N
M
Oc
G
GER
N
N
Og
10

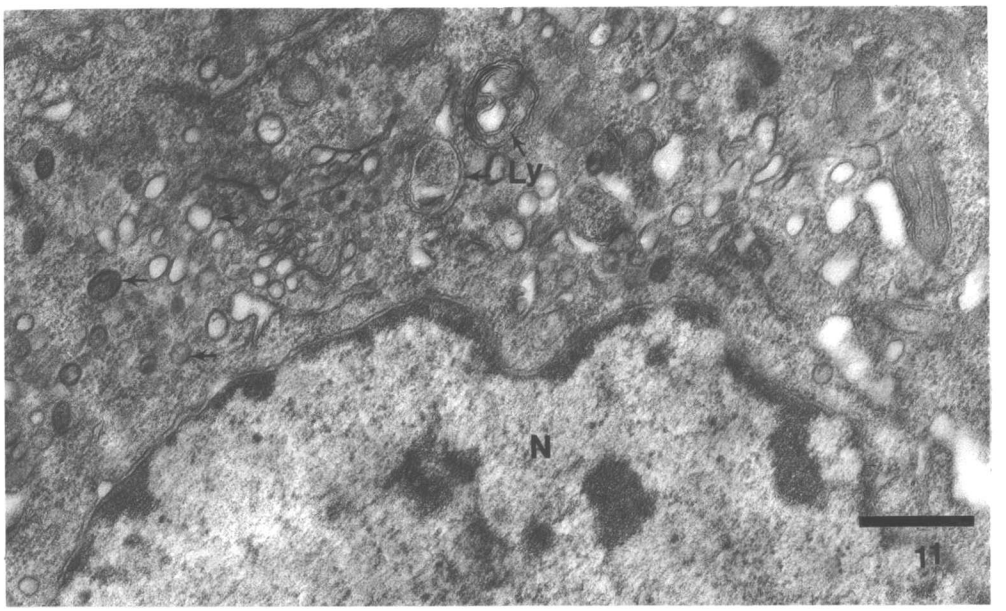
filled with either flocculent material or dark inclusions (Figs. 10 and 11).

In the posterior region of the ovary, the oocytes are more loosely packed and a continuous morphological series can be traced between the smaller oocytes at the periphery and the large, central ova (Fig. 12). The nuclei of these oocytes have dispersed chromatin masses and, occasionally, a single nucleolus. Besides the increase in size, the most conspicuous feature in the maturation of the oocyte is the presence of electron-dense bodies immediately beneath the plasma membrane. Higher magnification of these bodies, approximately 0.21 μm in diameter, indicated that they are membrane-bound structures containing small granules (Fig. 13).

In contrast to developing oocytes, the nuclei of mature oocytes contain sparse dense chromatin masses and a single, well-developed nucleolus (Fig. 14). These oocytes are in contact with a number of intercellular areas, which are filled with an amorphous material (Fig. 15). The cytoplasm contains numerous, dispersed, free ribosomes and mitochondria, few Golgi complexes, a sparse endoplasmic reticulum, nucleolus-like bodies near the nucleus (Figs. 16, 17 and 18) and several

Fig. 11. Part of a developing oocyte showing aggregations of vesicles (arrows). Ly, lysosome; N, nucleus. Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.5 µm.

Fig. 12. Section of the posterior region of the ovary showing a continuous morphological series of oocyte development. PO, peripheral oocyte; CO, central ovum; N, nucleus; Nu, nucleolus. Note the peripheral arrangement of cortical granules (arrows). Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 2.58 µm.


Ly
N
11

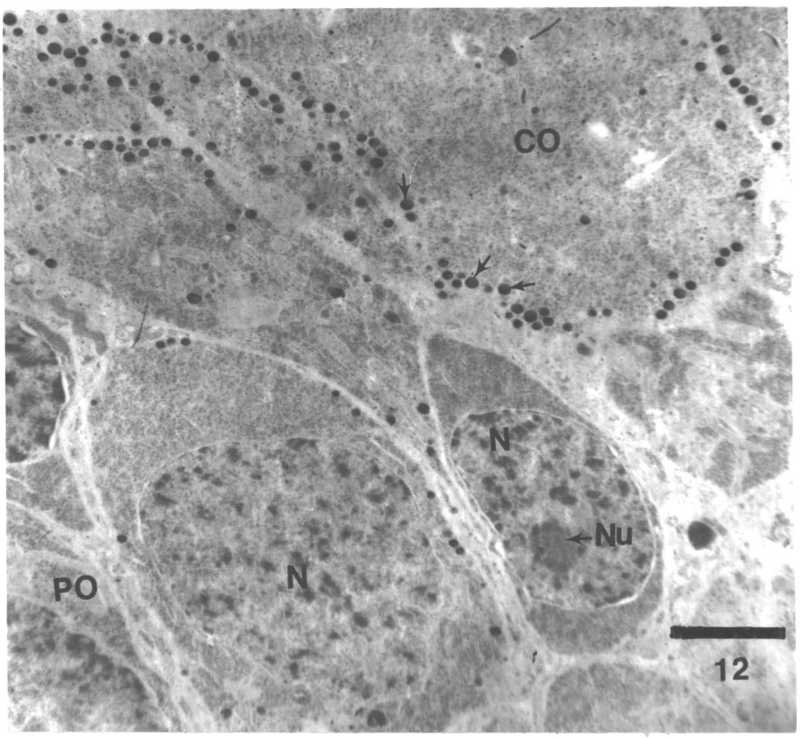

CO
N
Nu
PO
N
12

Fig. 13. Higher magnification of the area between the arrows in Fig. 12 illustrating the cortical granules (CG) beneath the plasma membrane (PM). Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 0.25 μm.

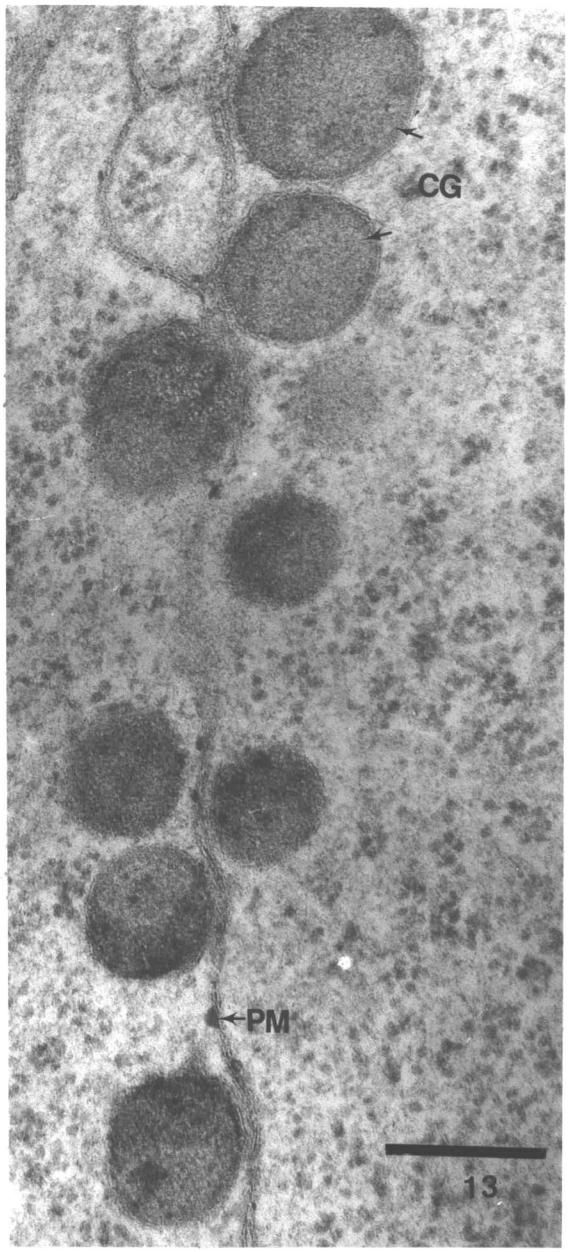

CG
PM
13

Fig. 14. Portion of a mature oocyte illustrating a well-developed nucleolus (Nu) and dispersed chromatin in the nucleus (N). Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Milloning buffer (H 7.2, 176 mOsm). Scale bar = 0.96 µm.

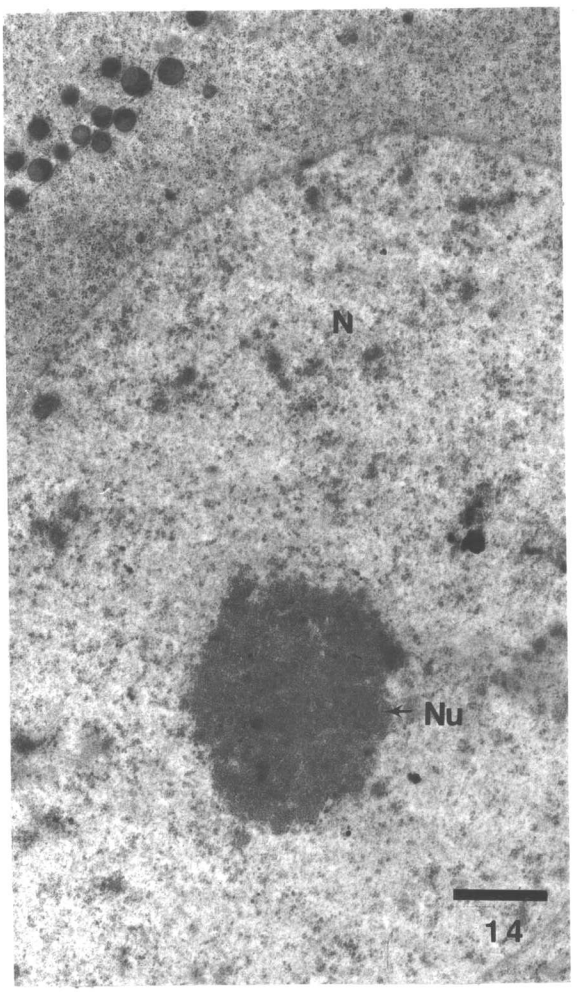

N
Nu
1.4

Fig. 15. Posterior region of the ovary showing an oocyte in contact with intercellular spaces (ICS). N, nucleus; Nu, nucleolus. Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, 176 mOsm). Scale bar = 1.19 µm.

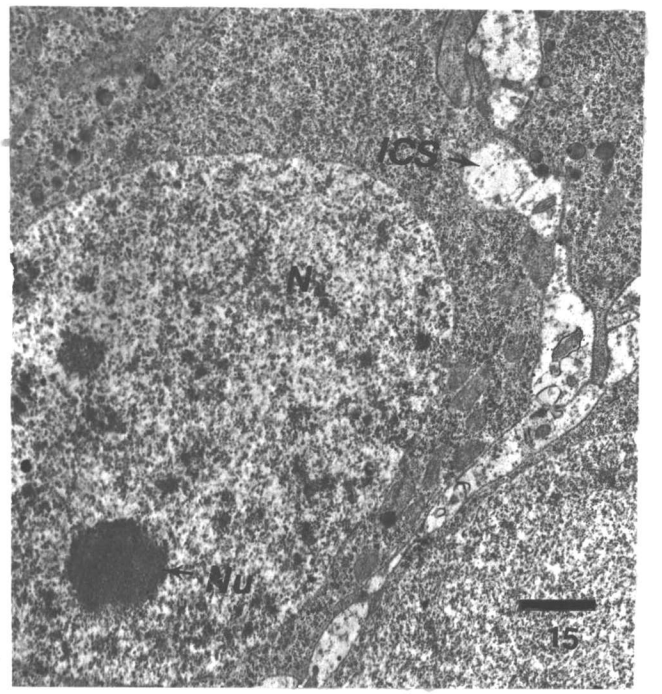

ICS
N
Nu
15

Fig. 16. Cross section of a mature oocyte (Oc) at the origin of the oviduct showing a sparse endoplasmic reticulum (arrows) and Golgi complex (G). S, sperm; M, mitochondrion. Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.97 μm.

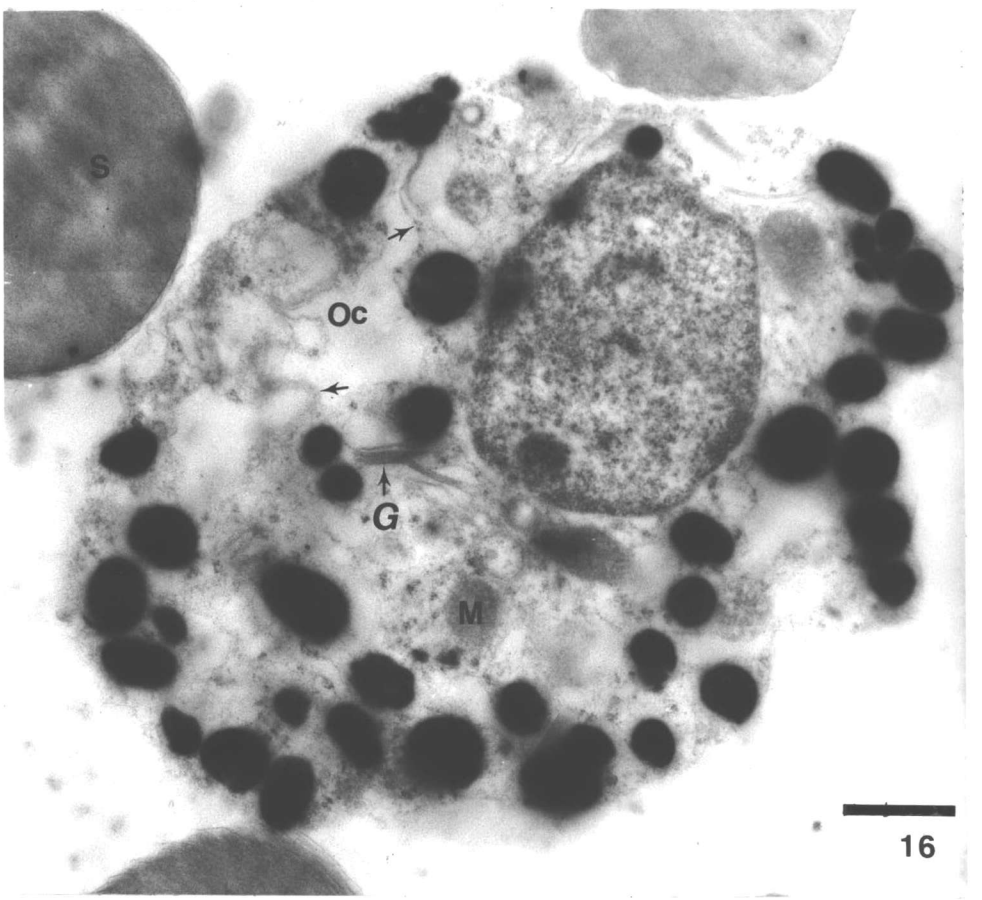
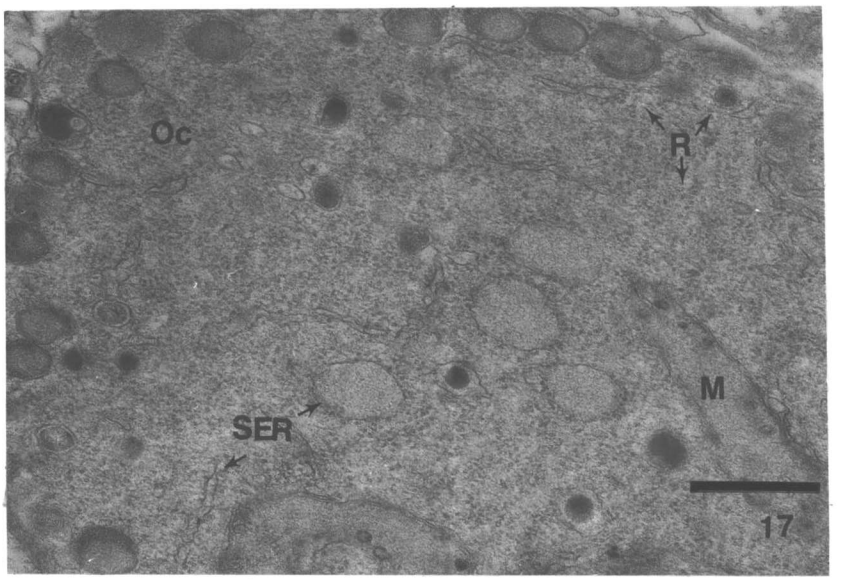
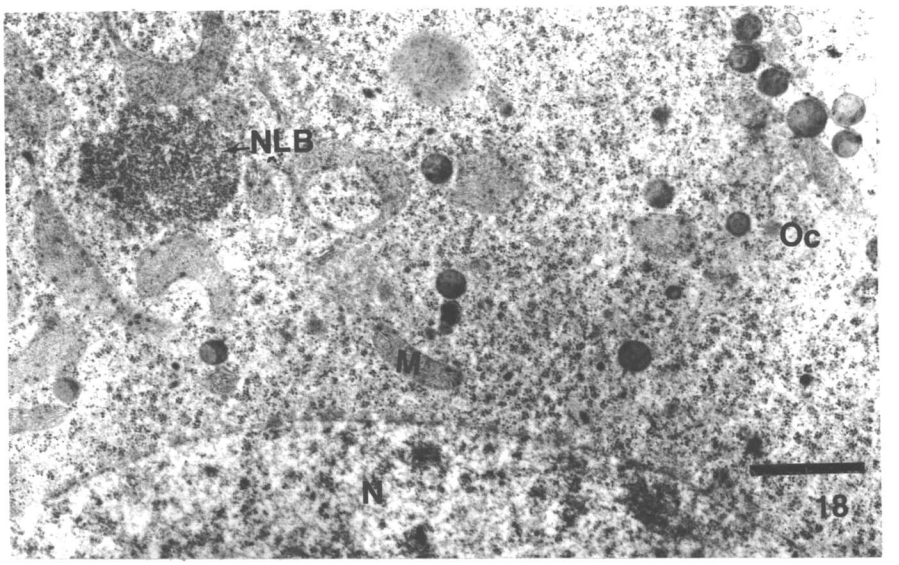

S
Oc
G
M
16

Fig. 17. Longitudinal section of a mature oocyte (Oc) showing smooth endoplasmic reticulum (SER), dispersed ribosomes (R) and mitochondria (M). Fixative: 3% glutaraldehyde in Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.5 μm.

Fig. 18. Perinuclear region of a mature oocyte (Oc) showing the nucleolus-like body (NLB) in close proximity to the nucleus (N) and mitochondria (M). Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, 176 mOsm). Scale bar = 1 μm.


Oc
R
M
SER
17


NLB
Oc
M
N
18

microtubular tracts near the periphery (Fig. 19). The endoplasmic reticulum occurs as fine, undilated strands which appear as slender, smooth tubules and small vesicles. The mitochondria contain few cristae and several dense granules. The nucleolus-like bodies consist of dense particles resembling ribosomes, approximately 150-200 Å in diameter, and are not delimited by membranes (Fig. 20). These bodies are in close proximity to mitochondria.

Ultrastructure of the Vitellaria

The vitelline follicles contain cells in various stages of maturation. The developing cells contain large nuclei, numerous free ribosomes and mitochondria. These cells are characterized by extensive parallel arrays of a dilated granular endoplasmic reticulum and large, well-formed Golgi complex throughout the cytoplasm (Figs. 21 and 22). The Golgi complexes are associated with numerous granular globules consisting of shell protein. These globules occur as single microvesicles of various sizes, which appear to coalesce (Figs. 23 and 24).

During maturation, the vitelline cells increased in size and the nuclei became more eccentrically located.

Fig. 19. Portion of the peripheral region of a mature oocyte illustrating microtubules (arrows) and mitochondria (M). Fixative: 3% glutaraldehyde in 100 mM Milloning buffer (pH 7.2) with 5% glucose. Scale bar = 0.5 μm.

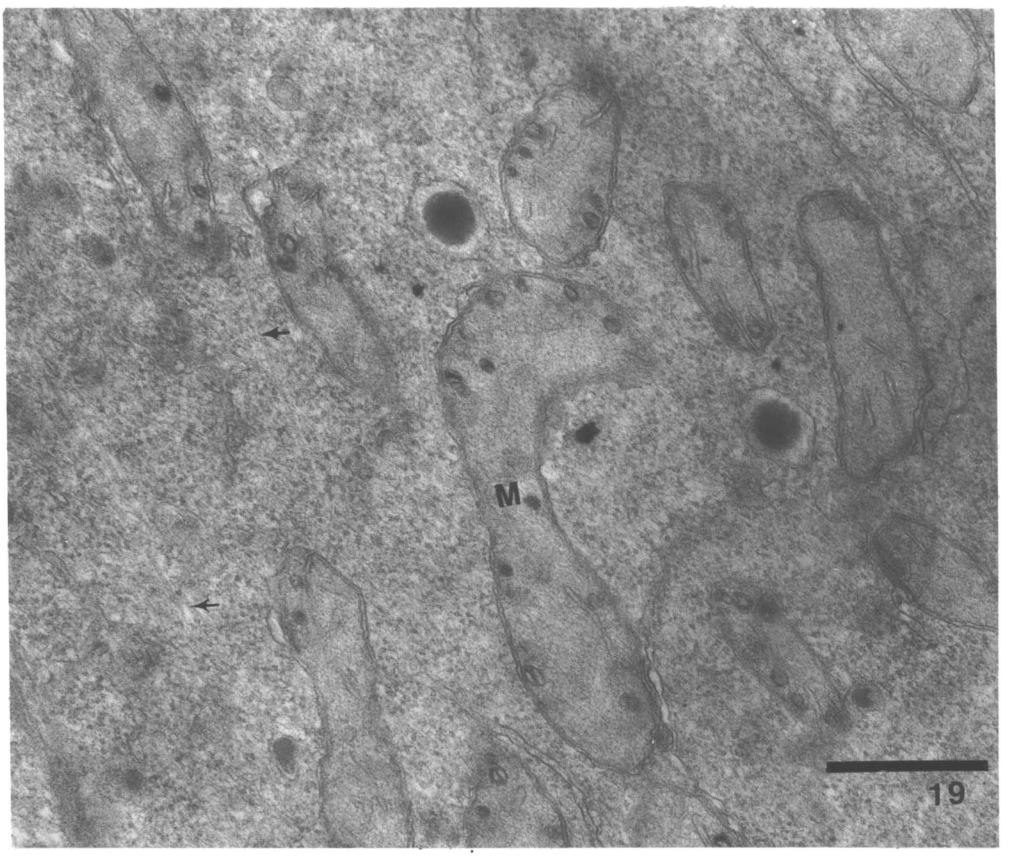

M
19

Fig. 20. Higher magnification of the perinuclear region of a mature oocyte demonstrating the nucleolus-like body (NLB) near mitochondria (M). Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, 176 mOsm). Scale bar = 0.25 μm.

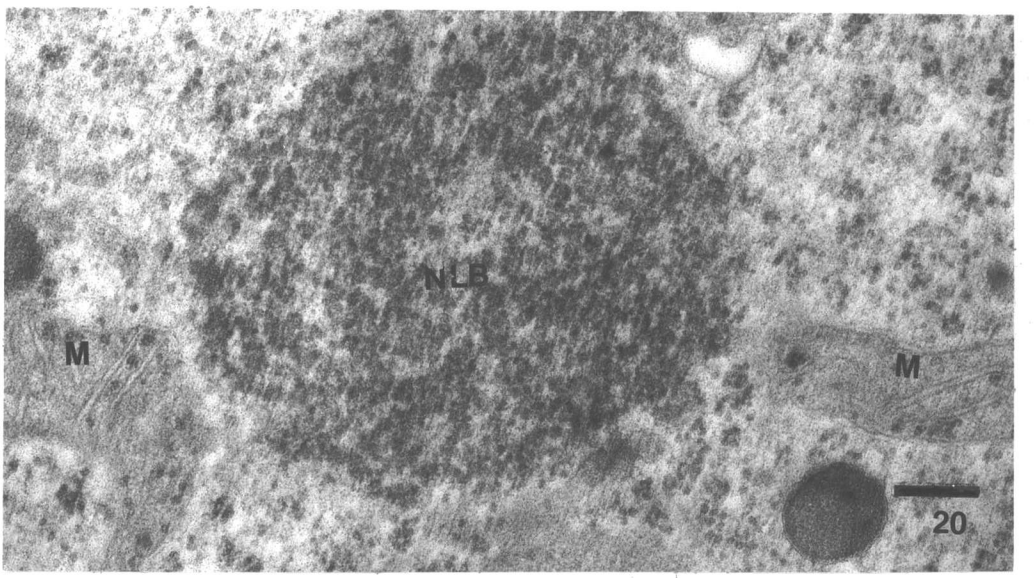
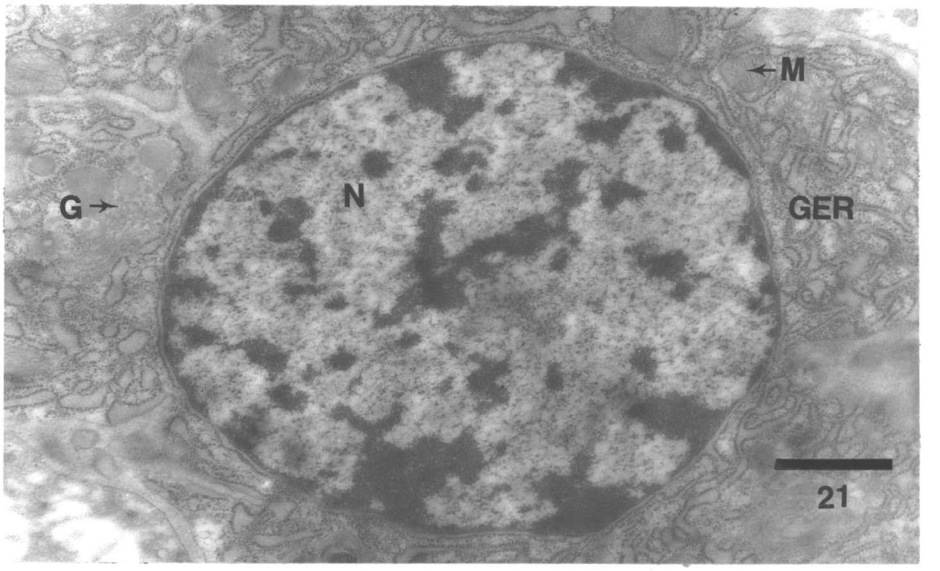

NLB
M
M
20

Fig. 21. Cross section of a developing vitelline cell showing a large central nucleus (N), mitochondria (M), well-developed granular endoplasmic reticulum (GER) and Golgi complex (G). Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.5 μm.

Fig. 22. Higher magnification of a portion of the developing vitelline cell shown in Fig. 21 demonstrating granular endoplasmic reticulum (GER) and Golgi complex (G) in association with synthesis of shell globules (SG). Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.5 μm.


M
G
N
GER
21

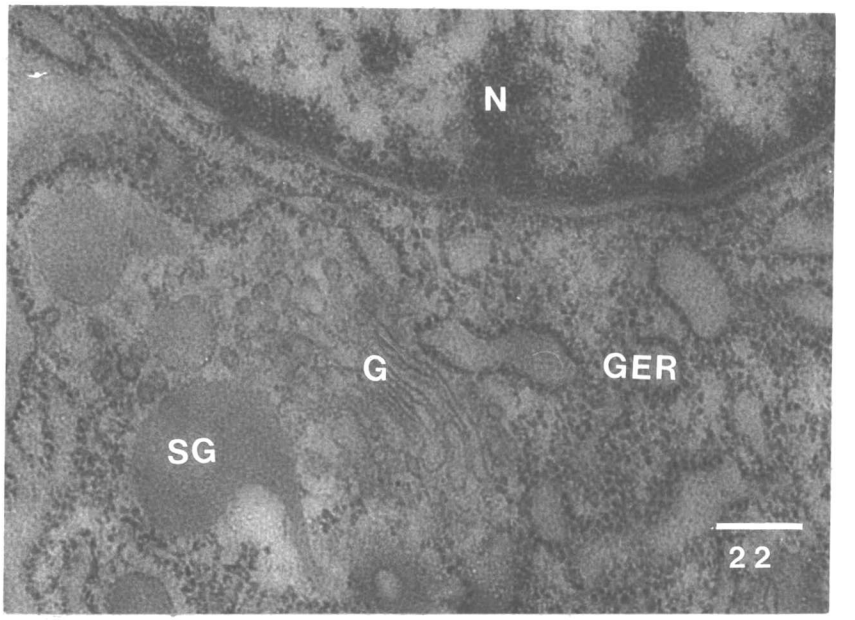
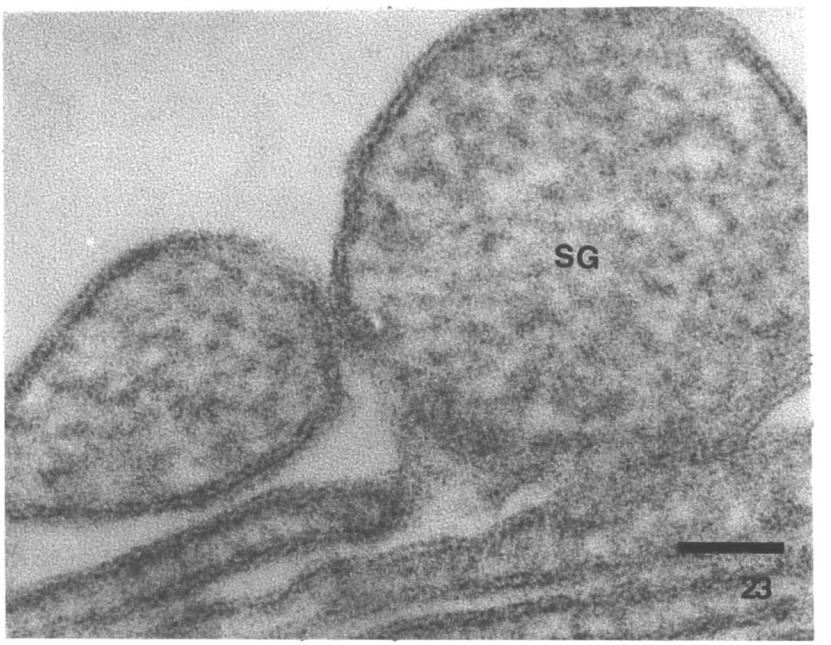
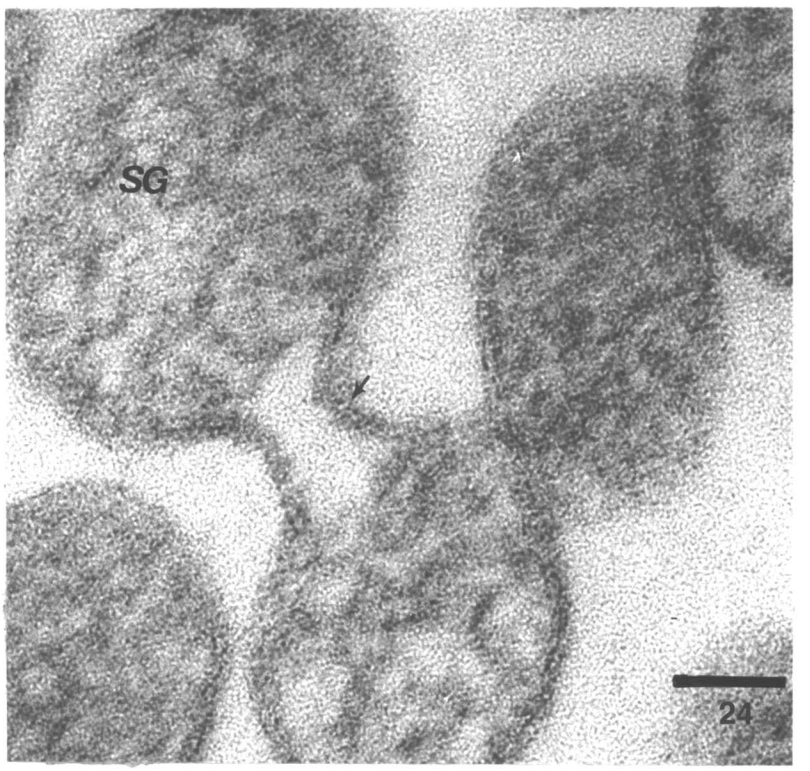

N
G
GER
SG
22

Fig. 23. Portion of a vitelline cell showing individual shell globules (SG). Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, 176 mOsm). Scale bar = 50 nm.

Fig. 24. Portion of a vitelline cell showing shell globules (SG) in the process of coalescing. The arrow indicates a protoplasmic bridge. Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, 176 mOsm). Scale bar = 50 nm.


SG
23


SG
24

Within the cytoplasm, numerous free ribosomes and mitochondria persist. Additional features, not seen in the young cells, include a large number of lipid droplets associated with the smooth endoplasmic reticulum, large membrane-bound clusters of shell globules, microtubules and scattered whorls of the endoplasmic reticulum (Figs. 25, 26a, 26b and 27). The parallel arrays of the granular endoplasmic reticulum are limited to the peripheral regions. Distinct Golgi complexes were rarely observed in these cells.

In the most advanced stage of development, mature cells are characterized by a concentration of large clusters of shell globules near the plasma membrane (Fig. 28). While parallel arrays of the granular endoplasmic reticulum are displaced peripherally, whorls of the granular endoplasmic reticulum are distributed throughout the cytoplasm and persist in vitelline cells within the egg capsules (Fig. 29). In addition to an abundance of lipid droplets, the cytoplasm contains copious amounts of dense granules resembling glycogen (Fig. 30). Preliminary histochemical tests indicated that these cells gave positive reactions when stained with periodic acid/Schiff (PAS) or Sudan black.

Fig. 25. Longitudinal section of a mature vitelline cell showing lipid droplets (L) and an eccentrically located nucleus (N). Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 1 µm.

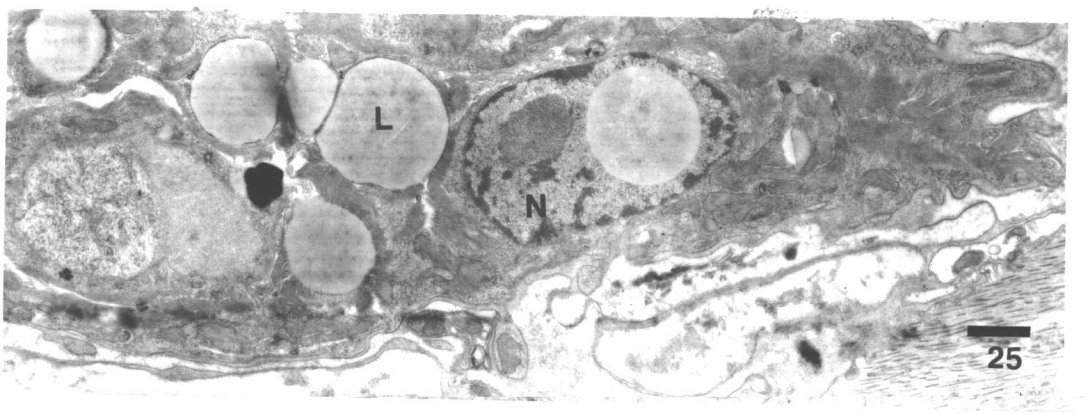
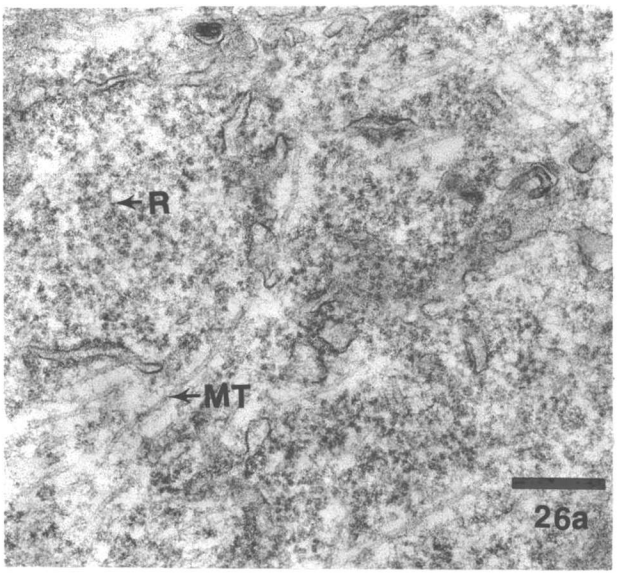

L
N
25

Fig. 26a. Region of the vitelline cell shown in Fig. 25 illustrating microtubules (MT) and free ribosomes (R). Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.96 µm.

Fig. 26b. Region of the vitelline cell shown in Fig. 25 illustrating smooth endoplasmic reticulum (SER) associated with lipid droplet (L). Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.96 µm.


R
MT
26a

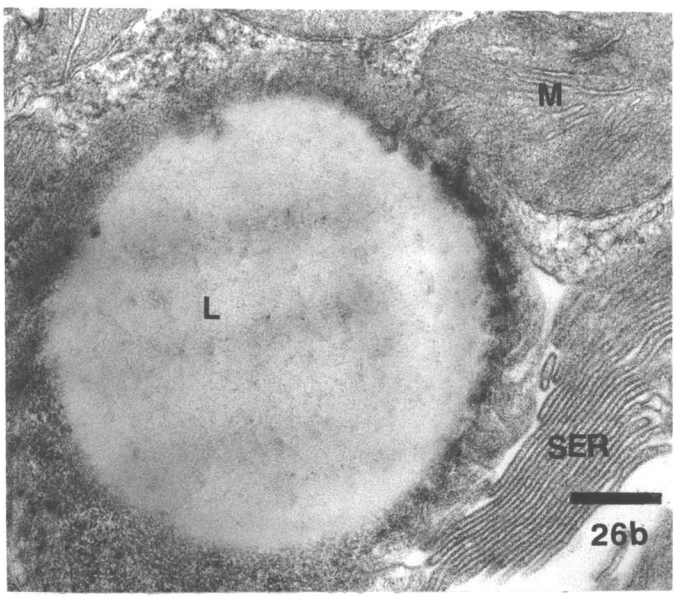
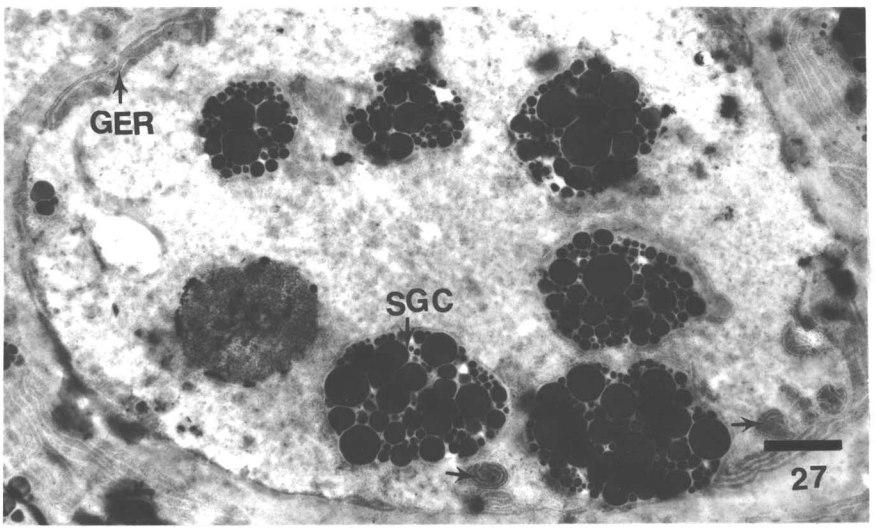

M
L
SER
26b

Fig. 27. Cross section of a vitelline cell in an advanced stage of development demonstrating granular endoplasmic reticulum (GER) and clusters of shell globules (SGC). The arrow indicates a whorl of granular endoplasmic reticulum. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 1 μm.

Fig. 28. Cross section of a mature vitelline cell illustrating clusters of shell globules (arrows) beneath the plasma membrane. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 1.72 μm.


GER
SGC
27

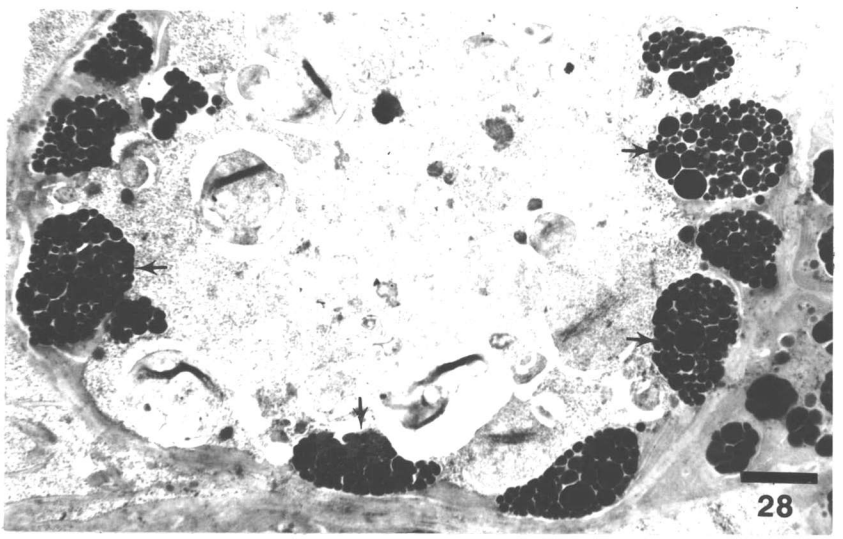

28

Fig. 29. An encapsulated egg showing whorls of granular endoplasmic reticulum (GER). ES, eggshell. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 0.25 µm.

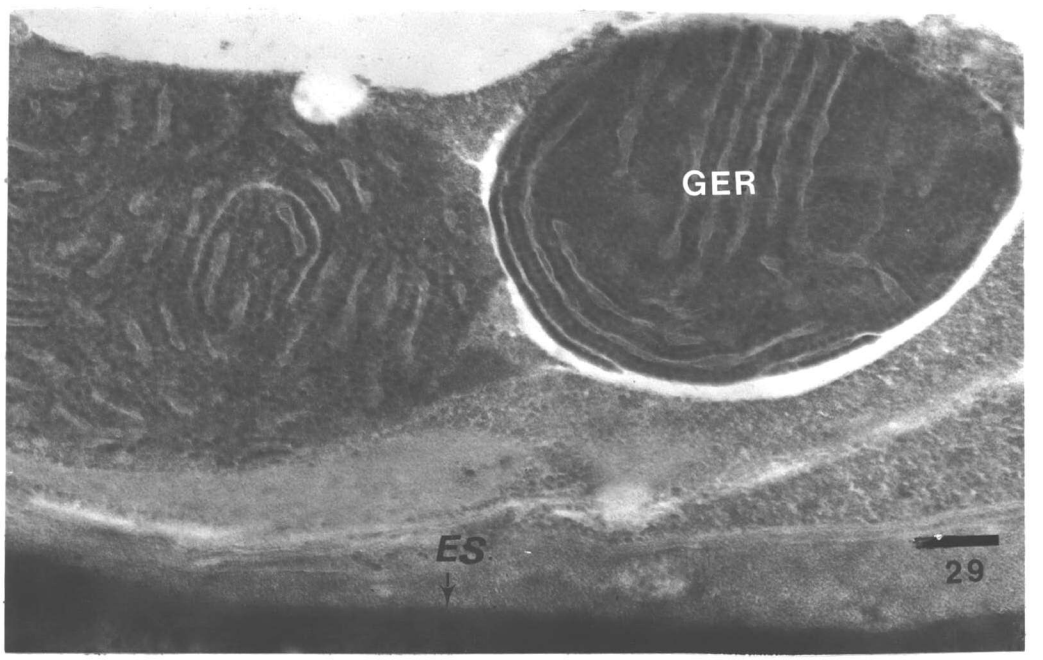
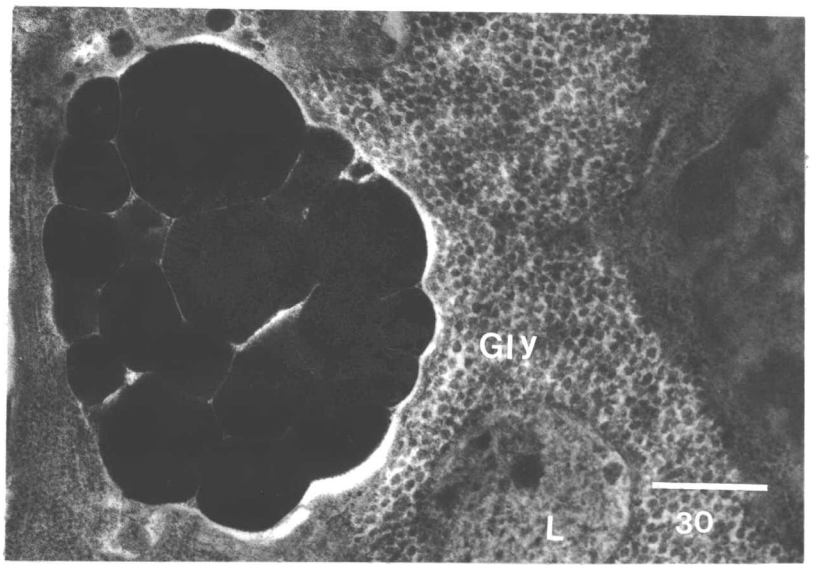

GER
ES
29

Fig. 30. Portion of a mature vitelline cell showing glycogen granules (Gly) and a lipid droplet (L). Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 0.5 μm.


Gly
L
30

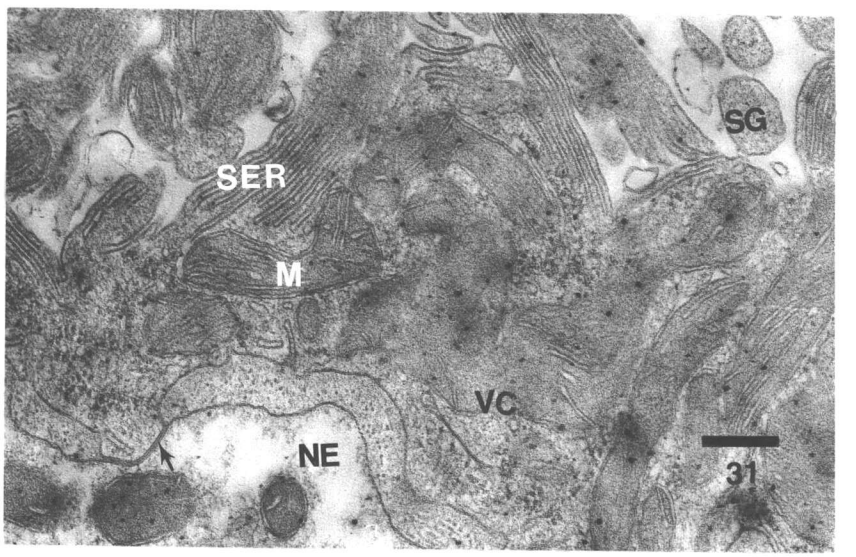
The vitelline follicles also contain a number of nurse cells. Junctional complexes frequently occur between cytoplasmic extensions of nurse cells and vitelline cells which produce large quantities of shell protein (Fig. 31 and 32).

Ultrastructure of the Encapsulated Egg

Dense body cells of Mehlis' gland empty their contents via processes which penetrate the epithelium of the ootype, whose long slender lamellae extend into the lumen of this structure (Figs. 33 and 34). Membranous body (MB)cells are posterior to this region. As seen in Figs. 35 and 36, the MB-cells contain secretory bodies which appear to be derived from expanded ends of Golgi complexes containing electron-dense material. In Fig. 37, portions of an ovum and spermatozoon are seen in the lumen of the ootype where membranous bodies have been secreted. No shell was apparent at this point. Closer examination of these membranous bodies show that they vary in appearance. In sagittal sections of the ootype, the membranous bodies are seen as stacks of closed sacs with some density along their inner surfaces (Figs. 38 and 39). In transverse sections, the membranous bodies are seen as ovoid structures

Fig. 31. A developing vitelline cell (VC) and surrounding cytolasmic extension of a nurse cell (NE) illustrating junctional complex (arrow). SG, shell globule; M, mitochondrion; SER, smooth endoplasmic reticulum. Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, 176 mOsm). Scale bar = 0.25 µm.

Fig. 32. Higher magnification of a junctional complex (arrows) between a vitelline cell (VC) and cytoplasmic extension of a nurse cell (NE). G, Golgi complex. Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, 176 mOsm). Scale bar = 0.1 µm.


SG
SER
M
VC
NE
31

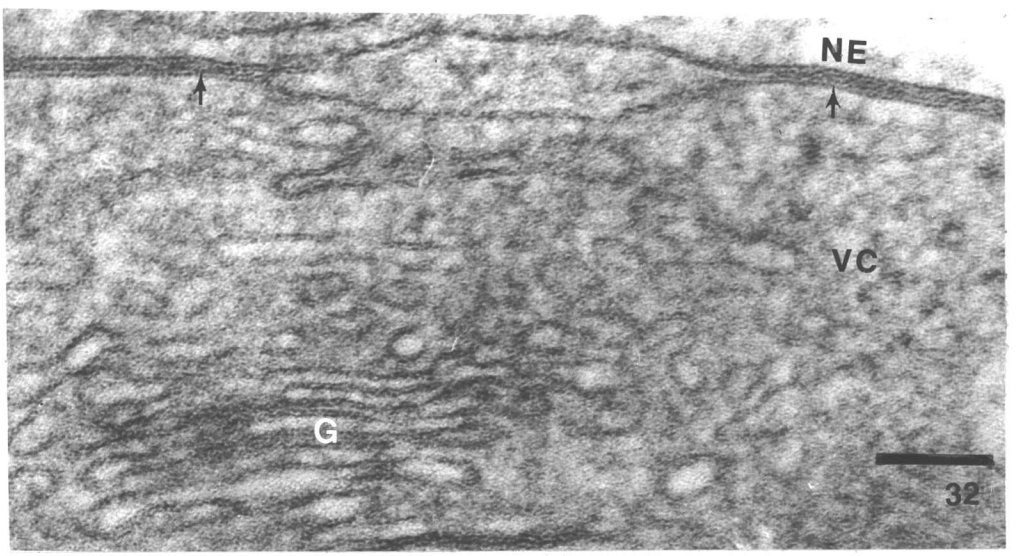
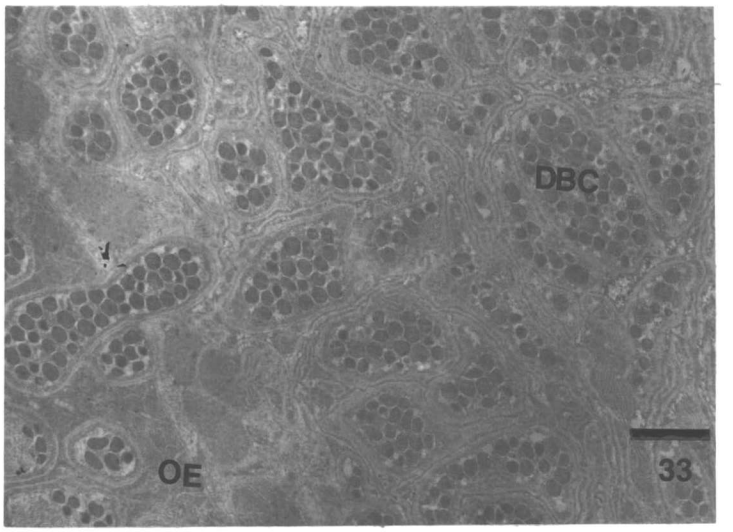

NE
VC
G
32

Fig. 33. Cross section of the Mehlis' gland showing a process (arrow) of a dense body cell (DBC) which has penetrated the epithelium of the ootype (OE). Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 1.38 µm.

Fig. 34. Longitudinal section of the ootype showing its epithelium (OE) and the process of a dense body cell (DBP). The arrows indicate lamellae of the ootype epithelium. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 0.5 µm.


DBC
OE
33

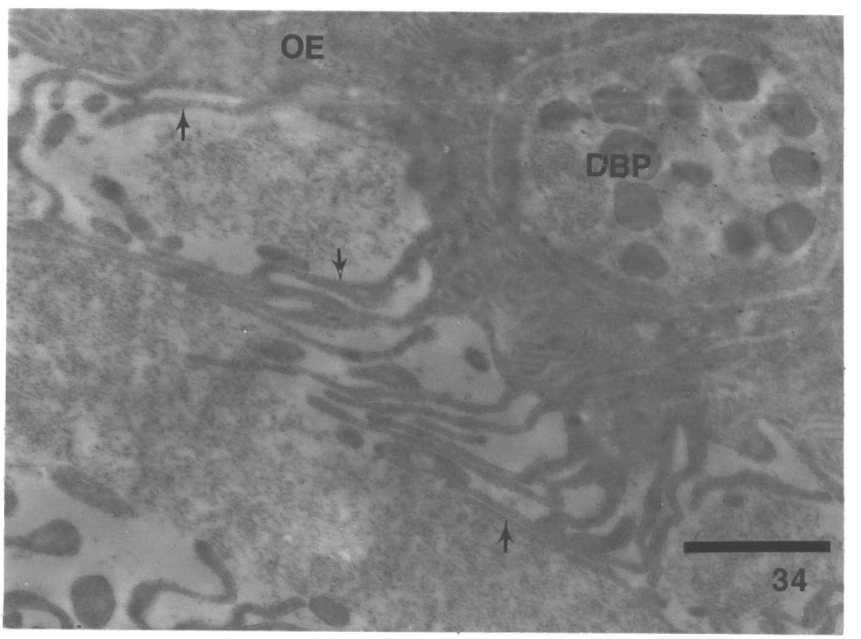
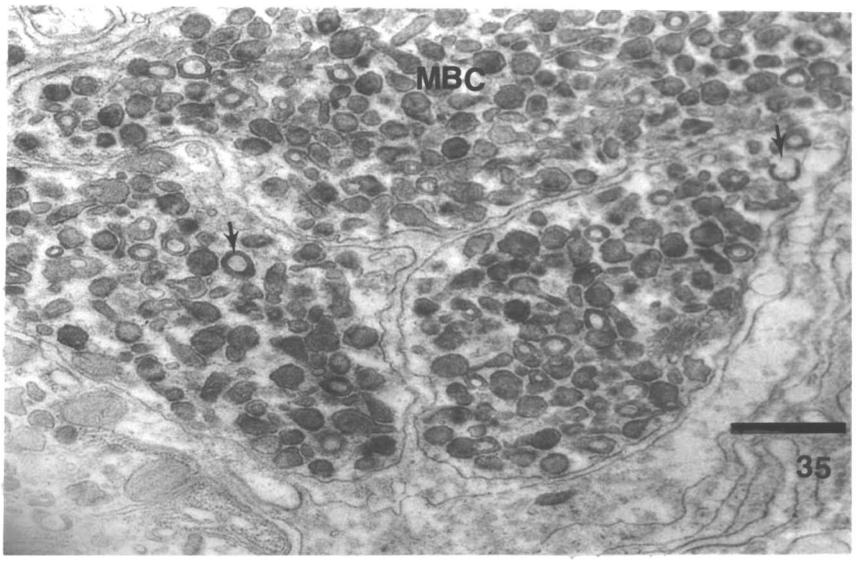

OE
DBP
34

Fig. 35. Portions of the Mehlis' gland showing the membranous body cells (MBC). The arrows indicate secretory products. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 1 µm.

Fig. 36. Higher magnification of a membranous body cell of the Mehlis' gland showing Golgi-associated vesicles (GV) with secretory products (arrows). Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 0.2 µm.


MBC
35

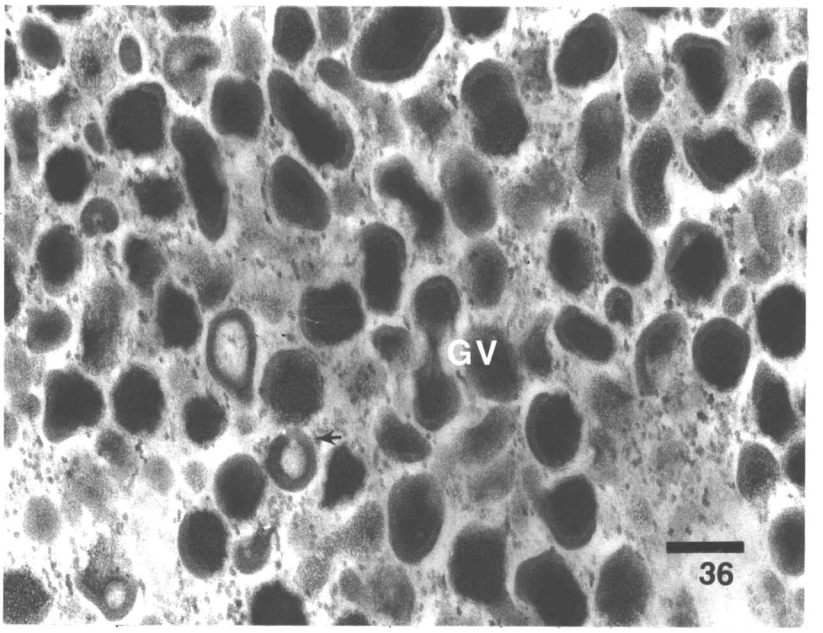

GV
36

Fig. 37. Longitudinal section of the ootype showing portions of an oocyte (Oc) and a sperm (S). OE, epithelium of the ootype; BL, basal lamina; CM, circular muscle. Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 1 μm.

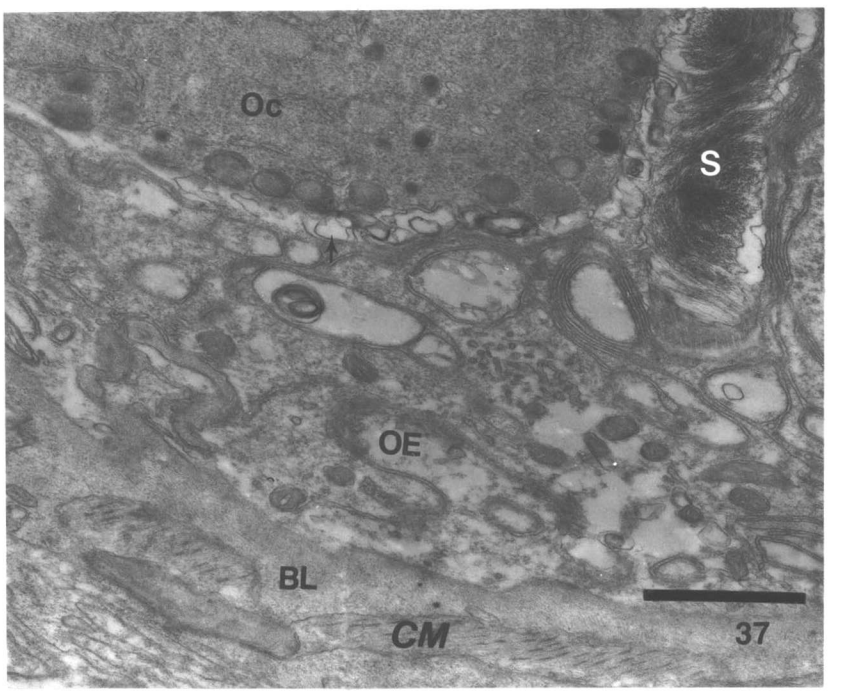
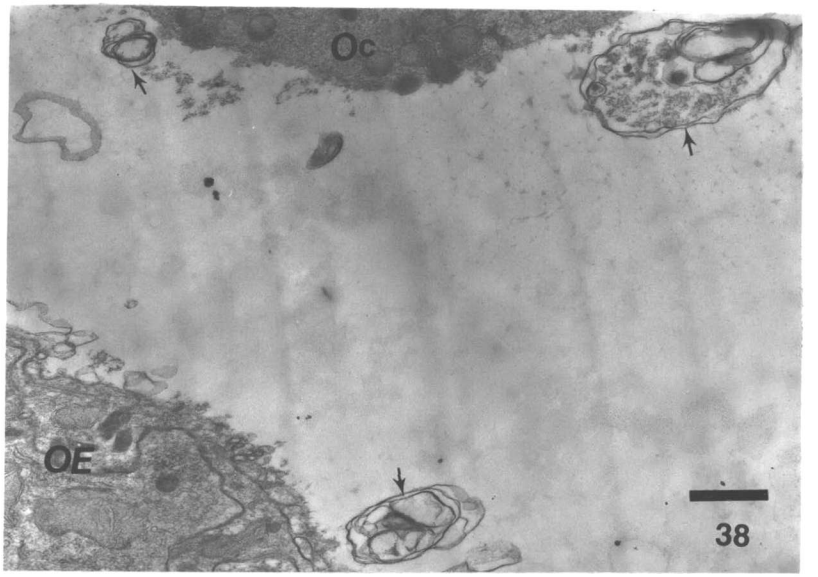
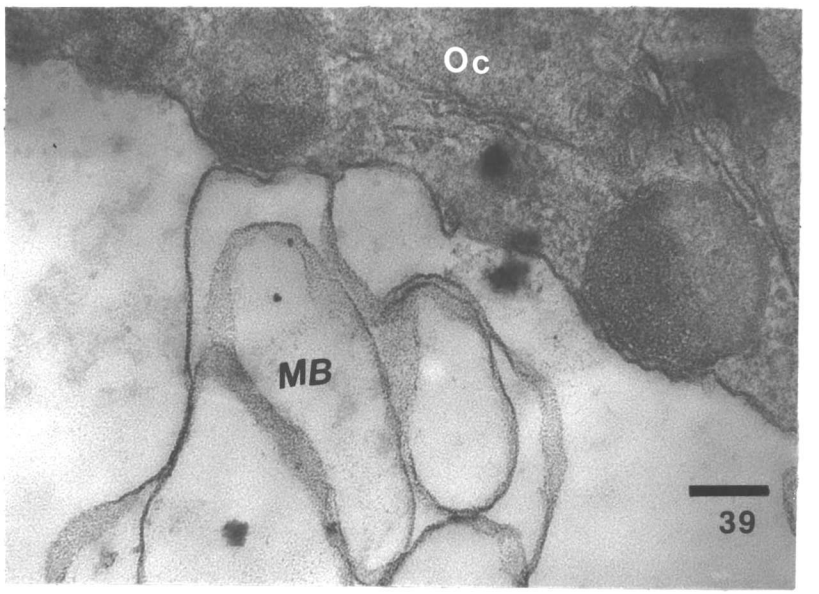

Oc
S
OE
BL
CM
37

Fig. 38. Membranous bodies (arrows) and an oocyte (Oc) in the ootype. OE, epithelium of the ootype. Fixative: 3% glutaraldehyde in 100 mM Millonig buffer with 5% glucose. Scale bar = 1 µm.

Fig. 39. Membranous body (MB) at the surface of an oocyte (Oc) in the lumen of the ootype. Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.25 µm.


Oc
OE
38


Oc
MB
39

containing strands of dense material radiating from a central core (Fig. 40).

Incomplete eggshells were not seen in this study. However, completely encapsulated eggs were observed in the more distal region of the ootype and in the proximal uterus, where the cytoplasmic extensions of MB-cells continued to open into the lumen for some distance (Fig. 41). The eggshell consists of a relatively thick layer of shell protein, approximately 1.5-2.0 μm in diameter, and is very electron-dense. At higher magnification, a layer of reticular secretion is seen to cover the outer surface of young eggs in the ootype (Fig. 42). Membranous areas with concentric lamellae are associated with the reticular substance.

Just beyond the region shown in Fig. 42, residual reticular secretions are seen on the inner and outer surfaces of the eggshells of young eggs in the proximal uterus (Figs. 43a and 43b). These egg capsules appear to be similar, in terms of thickness and electron-density, to those in the distal ootype.

When compared to eggs in the ootype-proximal uterus, older ones in the distal uterus showed a number of changes. In addition to an increase in diameter,

Fig. 40. Cross section view of membranous bodies in the lumen of the ootype illustrating the cores of these bodies (arrows). ci, cilia projecting from the posterior region of the oviduct. Fixative: 3% glutaraldehyde in 100 mM Millonig buffer (pH 7.2) with 5% glucose. Scale bar = 0.25 µm.

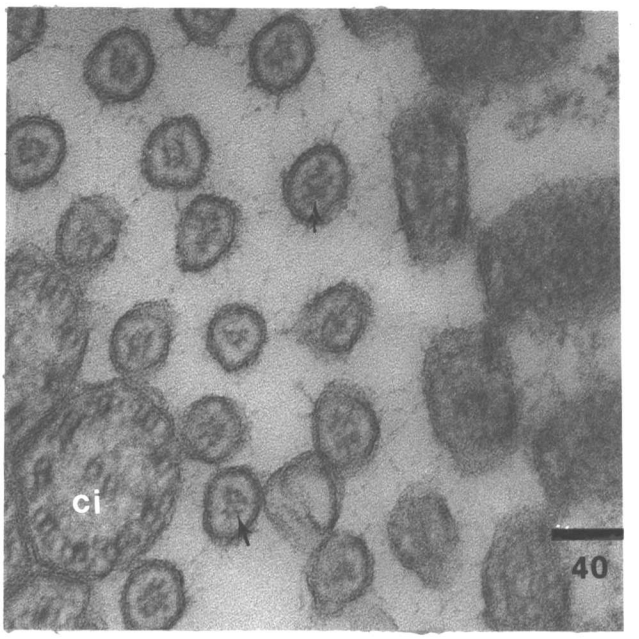

ci
40

Fig. 41. Longitudinal section showing a membranous body cell (MBC) and an encapsulated egg in the ootype. ES, eggshell; OE, epithelium of the ootype. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM Millonig buffer (pH 7.3) with 10 mM sucrose. Scale bar = 2.08 µm.

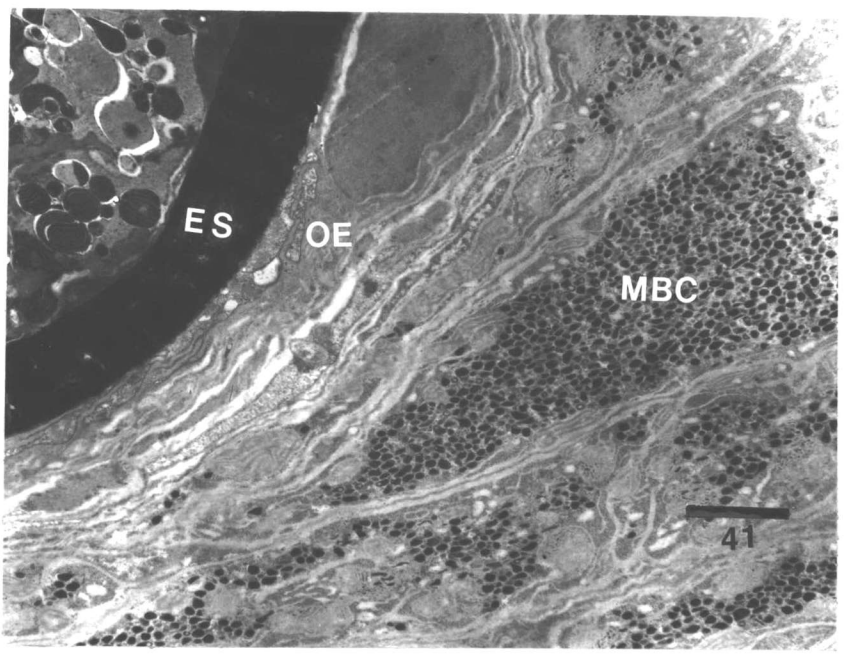
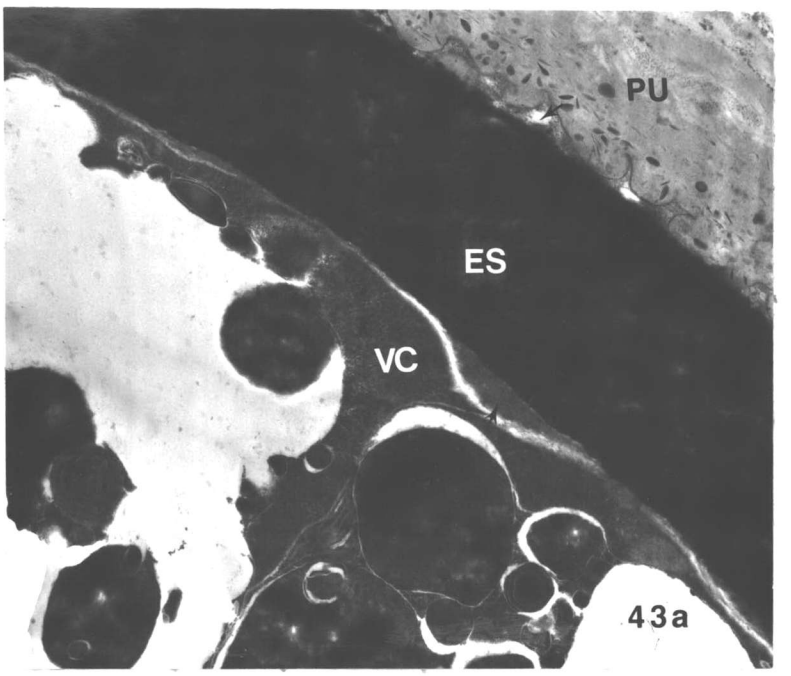

ES
OE
MBC
41

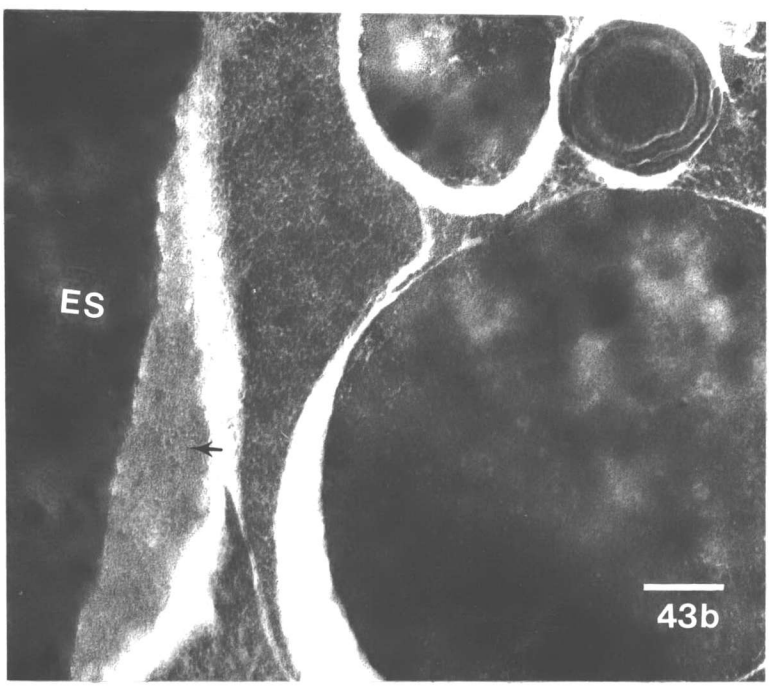
Fig. 42. Encapsulated egg surrounded by the secretory product (S1) of the membranous body cells. ES, eggshell; Lm, lamellae. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 0.1 μm.


Lm
S1
ES
42

Fig. 43a. Encapsulated egg with residual S1 secretion (arrows) in the proximal uterus (PU). ES, eggshell; VC, vitelline cell. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 0.82 µm.

Fig. 43b. Higher magnification of the area indicated by the arrow between the vitelline cell and eggshell in Fig. 43a. ES, eggshell; arrow, reticular secretion. Fixative: 5% glutaraldehyde, 4% paraformaldehyde and 360 mM DMSO in 30 mM sodium cacodylate buffer (pH 7.3) with 10 mM sucorse. Scale bar = 0.25 µm.


PU
ES
VC
43a


ES
43b

approximately 7-8 μm, these eggshells are notably less dense and, in many case, relatively large spaces occur between the shell and its contents (Fig. 44). At higher magnification, older shells appear to be blotched with small, electron-lucid areas (Fig. 45). Some of these areas contain finely granular cores. A layer of electron-dense secretion is seen associated with eggshells in the distal uterus (Fig. 46). This layer consists of small clusters of round, dense granules.

Fig. 44. Encapsulated eggs in the distal uterus demonstrating electron-lucid areas in the eggshell. The arrow shows a large space between the eggshell and contents of the egg. Fixative: 1% glutaraldehyde and 4% paraformaldehyde in Milloning buffer (pH 7.2, 176 mOsm). Scale bar = 1.03 µm.

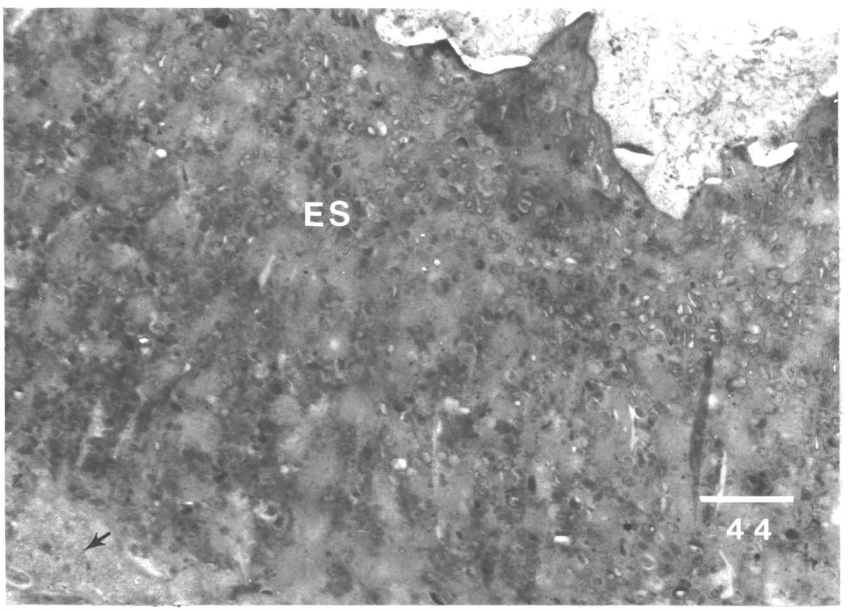

ES
44

Fig. 45. Higher magnification of an eggshell (ES) showing electron-lucid areas with finely granular cores (arrows). Fixative: 1% glutaralehyde and 4% paraformaldehyde in Millonig buffer (pH 7.2, 176 mOsm). Scale bar = 2.1 μm.

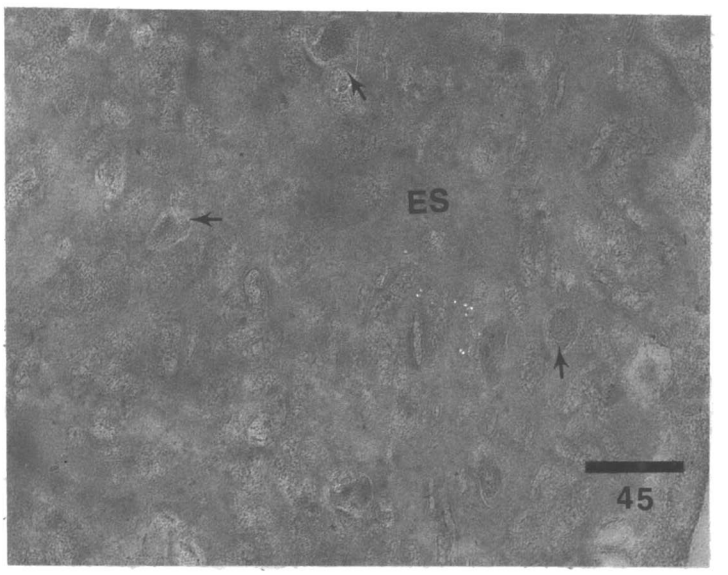
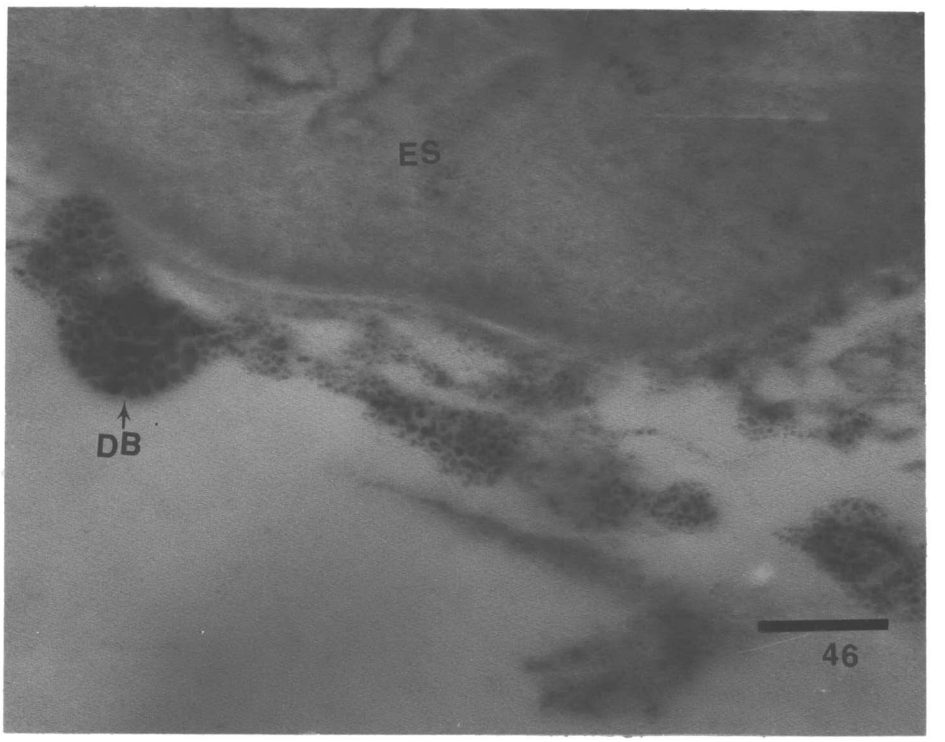

ES
45

Fig. 46. Portion of an encapsulated egg in the distal uterus illustrating dense bodies (DB) on the outer surface of the eggshell (ES). Fixative: 5% glutaraldehyde, 4% parformaldehyde and 360 mM DMSO in sodium cacodylate buffer (pH 7.3) with 10 mM sucrose. Scale bar = 88 nm.


ES
DB
46

CHAPTER V

DISCUSSION

Histochemical Localization of Shell Precursors

The histochemical results of sectioned material in the present investigation showed that the components of the quinone-tanning system, i.e. basic proteins, phenols and phenolase were specifically localized in the shell globules of vitelline cells and eggshells of Acanthoparyphium spinulosum. Fried and Stromberg (1971) reported that all three components were histochemically demonstrated in vitellaria and eggshells of other echinostomes, namely Echinostoma revolutum and Echinoparyphium recurvatum. These results are also in accord with recent histochemical studies by Gupta and Puri (1981) on eggshell formation in Fasciola indica and Fasciola buski.

Although the malachite green and bromophenol blue methods demonstrated basic proteins in the vitelline cells and eggshells, a difference in affinity for these dyes was discerned in these structures. This observation is consistent with previous studies in Fasciola by Smyth and Clegg (1959) in which malachite green was reported to be held tightly by granules within the shell

globules giving them a mulberry-like appearance. Bromophenol blue, however, was not held by the shell globules as tightly as malachite green and the mulberry pattern was barely detected.

In the present investigation, neither malachite green nor bromophenol blue stained the testes and ovary of Acanthoparyphium spinulosum. Shropshire (1984) used whole mounts of A. spinulosum and reported, however, moderate reactions for malachite green in these organs, indicating the presence of basic proteins. Similarly, Boyer (1972) reported that oocytes of a polyclad turbellarian, Prostheceraeus floridans, strongly reacted for basic proteins when mercuric bromophenol blue was used. Moreover, Nollen (1973) reported that autoradiographic techniques did not indicate selective incorporation of tyrosine into vitellaria of Gorgoderina attenuata. Based on histochemical results from whole mounts of A. spinulosum reported by Shropshire (1984), it is possible that the results obtained in this study may reflect a difference in the thickness of sectioned tissues, when stained with malachite green or bromophenol blue.

While the results of the fast red salt B method in whole mounts and sections showed that the vitelline cells of A. spinulosum were strongly positive for phenolic substances, only the sectioned material showed a contrast in the staining of vitelline cells and eggshells. Similar observations were reported by Burton (1963) and Nollen (1971) in Haematoloechus medioplexus, with different azo-coupling methods. The observation that eggshells, especially the older capsules in the distal uterus, stained less intensely than the vitelline globules suggests that the phenols in the eggshells may have been undergoing oxidation to quinone in the tanning process.

The reddish-brown color, produced by incubation of sectioned material with catechol, appeared only in eggshells and shell globules in the vitellaria. The observation that surrounding tissues, including the testes and oviduct, did not produce a color reaction tends to suggest that the reaction resulted from enzyme action rather than from slow oxidation of catechol in solution. Smyth (1954) demonstrated the occurrence of phenolase in eggshell formation of a number of trematodes (Dolichosaccus rastellus, Cryptocotyle lingula,

Diclidophora merlangi and Haematoloechus sp.) and cestodes (Schistocephalus solidus and Proteocephalus filicollis), and in byssus formation of the mollusk, Mytilus edulis, with the catechol method.

Ultrastructure of the Ovary

The results of the electron microscopic examination of the ovary showed that oocyte differentiation involved a series of interconnecting events as follows: (1) the granular endoplasmic reticulum became organized and Golgi complexes associated with vesicles appeared in the cytoplasm; (2) the synthesis of cortical granules was initiated; (3) granular masses with no delimiting membranes, presumably nucleolus-like bodies (see Koulish, 1965), appeared in the cytoplasm near the nucleus; and (4) the cortical granules became localized around the periphery of the oocyte. This sequence of events is in accord with observations in other digenetic trematodes which have been reported by Bjorkman and Thorsell (1964), Erasmus (1973), Grant et al. (1977) and Justine and Mattei (1984). Moreover, according to Halton et al. (1976) and Boyer (1972), these events also occur during oogenesis in monogenetic trematodes and turbellarians.

In the maturation of oocytes, the development of the granular endoplasmic reticulum and the appearance of the Golgi complex were observed to be closely associated with the synthesis of cortical granules. Histochemical analysis of these granules in Fasciola hepatica by Bjorkman and Thorsell (1964) indicated that these granules were osmophilic, non-sudanophilic and polysaccharidic. Boyer (1972) reported that cortical granules in Prostheceraeus floridans contained basic proteins and neutral mucopolysaccharides. Shropshire (1984) demonstrated the presence of acidic mucopolysaccharides in the ovary of Acanthoparyphium spinulosum. The close structural relationship between the granular endoplasmic reticulum, Golgi complex and newly formed cortical granules suggests that the protein portion of the granule is synthesized in the granular endoplasmic reticulum and then complexes with polysaccharide in the Golgi complex. A similar scheme of events has been proposed for the synthesis of cortical granules in other flatworms (Boyer, 1972; Halton et al., 1976) and in organisms belonging to a number of different phyla (Balinsky and Devis, 1963; Anderson, 1968; Kessel, 1968).

In mature oocytes of A. spinulosum, the Golgi complex was rarely observed and the sparse endoplasmic reticulum appeared as smooth, undilated strands. Similarly, Halton et al. (1976) described the mature oocytes of monogeneans as having few or no Golgi complexes and a diminutive endoplasmic reticulum of two types. The latter either became reduced in dimensions by having few attached ribosomes, or it became fragmented and devoid of ribosomes. In this case, the endoplasmic reticulum appeared as smooth tubules and small, granular vesicles. Furthermore, diminution of the endoplasmic reticulum has been reported in Fasciola hepatica by Bjorkman and Thorsell (1964), in Schistosoma mansoni by Erasmus (1973) and in Haematoloechus medioplexus by Burton (1967a). The cytoarchitecture of the mature oocytes suggests that production of cortical granules ceases at the onset of maturation of ova, which is reflected in the diminution of the endoplasmic reticulum.

It was further noted that, in association with the cortical granules, several microtubular tracts were present in the periphery of mature oocytes. This is perhaps the first description of microtubular tracts in

oocytes of digenetic trematodes. However, reference to microtubules in the cytoplasm of oocytes in monogenetic trematodes has been made by Halton et al. (1976). The occurrence of these structures suggests that they may play a role in the intracellular transport of cortical granules to the periphery of the cell. Preston and Jenkins (1984) suggested a similar role for microtubules in the nematode, Trichuris muris.

In addition, it was observed that the mitochondria in primary oocytes of Acanthoparyphium spinulosum contained few cristae and several dense granules. These features have also been described by Bjorkman and Thorsell (1964) in Fasciola hepatica and by Grant et al. (1977) in Pharyngostomoides procyonis. Bjorkman and Thorsell (1962) suggested that the small number of cristae is correlated with the anaerobic conditions under which these parasites live. Bjorkman and Thorsell (1964) reported that the dense granules in mitochondria are similar to those in parenchymal cells of many invertebrate and vertebrate cell types. The significance of these granules, however, is not known.

A close association between the mitochondria and nucleolus-like bodies was observed in Acanthoparyphium

spinulosum. Such a relationship has been reported for primary oocytes of other organisms, such as ascidians (Hsu, 1963) and amphibians (Massover, 1968). Andre (1962) and Clerot (1968) suggested that the granules of the nucleolus-like body may participate in the synthesis of mitochondria; whereas, Eddy and Ito (1971) suggested that the mitochondria may provide energy that could transform or activate the granules into synthetic activity within the cell.

Finally, intercellular spaces, filled with an amorphous material, were observed in contact with primary oocytes in the posterior end of the ovary in A. spinulosum. Erasmus (1973) reported such intercellular spaces in contact with ova in Schistosoma mansoni and Gresson (1964b) reported these spaces in contact with most, if not all, the oogonia and primary oocytes in F. hepatica. It has been suggested that such a system of intercellular spaces is involved with transport between germ cells.

Ultrastructure of the Vitellaria

The vitelline follicle showed cells in various stages of maturation in Acanthoparyphium spinulosum. Developing cells contained an organized granular

endoplasmic reticulum and a well-formed Golgi complex, which were not seen in immature cells. Mature cells were characterized by large clusters of shell globules beneath the plasma membrane, numerous whorls of the endoplasmic reticulum and an abundance of lipid and glycogen. The sequence of developmental stages in A. spinulosum is very similar to ultrastructural aspects described by Halton et al. (1974) in vitelline cells of the monogeneans. With the exception of minor differences in the distribution of glycogen and lipid material, vitelline development in A. spinulosum is also similar to that described in a number of other digenetic trematodes (Irwin and Threadgold, 1970; Erasmus, 1973; Grant et al., 1977; Irwin and Maguire, 1979).

The occurrence of Golgi complexes and an organized granular endoplasmic reticulum in developing vitelline cells is consistent with the roles of vitelline cells, which are to produce shell material and provide nutrients for the fertilized ovum. The fact that the shell globules consist primarily of protein has been established in A. spinulosum by the histochemical methods in this study and those of Shropshire (1984). Further, the morphological data obtained in the present study

indicate that this protein is synthesized in the dilated cisternae of the endoplasmic reticulum and transferred to the abundant Golgi complexes, associated with shell globules, to be condensed and secreted. These data are supported by observations in Schistosoma mansoni by Erasmus (1975b), who reported that tritiated tyrosine was localized in the granular endoplasmic reticulum and vitelline globules.

It was also observed, in Acanthoparyphium spinulosum, that the appearance of whorls of the granular endoplasmic reticulum corresponded with the displacement of parallel cisternae to the periphery of mature vitelline cells. Irwin and Threadgold (1970) and Halton et al. (1974) described a similar morphological change in Fasciola hepatica and in monogeneans, respectively. Irwin and Threadgold (1970) observed that these whorls, referred to as so-called "yolk globules," were associated with abundant glycogen granules and bound by membranes, and suggested that they served as food reserves in F. hepatica. In A. spinulosum, however, there was not a close association between whorls of the granular endoplasmic reticulum and glycogen granules in vitelline cells. Although the abundant glycogen reserves may be

required by eggs of both species as they undergo developmental changes in an external aquatic environment, the function of whorls of the granular endoplasmic reticulum in A. spinulosum is not as apparent as in F. hepatica. It is probable that they serve as a source of nutrition. Erasmus (1973) suggested that they may have a greater physiological significance during the development of the egg in Schistosoma mansoni.

In addition, a number of cytoplasmic extensions of nurse cells were found around the vitelline cells in A. spinulosum. They frequently formed junctional complexes with cells in which large quantities of shell protein were synthesized. Irwin and Threadgold (1970) and Irwin and Maguire (1979) suggested a role of selection and transport of nutritive material for nurse cells in F. hepatica and Gorgoderina vitelliloba. It is possible that nurse cells in A. spinulosum may also have a similar role.

Ultrastructure of Encapsulated Eggs

Results obtained in this study showed that shell deposition in A. spinulosum occurred in that portion of the ootype-uterine tract which is penetrated by processes from the membranous body cells of the Mehlis'

gland. Although eggs in the more distal end of the ootype and proximal uterus were completely surrounded by a relatively thick layer of shell material, these egg capsules were notably thinner than those in the distal uterus. The difference in thickness of eggshells in these regions suggests that shell deposition continues in the uterus. Gonnert (1962) also reported observations which indicated that eggshell formation continued in the uterus of F. hepatica. Uterine involvement in shell formation has been reported in other trematodes (Bhatnagar and Gupta, 1984), including Syncoelium spathulatum by Coil and Kuntz (1963), Hydrophitrema gigantica by Coil (1965) and Ogmocotyle indica by Coil (1966). According to Coil and Kuntz (1963), the bulk of the eggshell in S. spathulatum is secreted by epithelial cells of the uterus. However, it is more likely that, in A. spinulosum, the increase in shell thickness occurs from shell deposits within the egg capsule as it passes along the uterus.

In A. spinulosum, the older capsules in the uterus were apparently less dense than those in the ootype and proximal uterus. They appeared to be blotched with small, electron-lucid areas, some of which contained

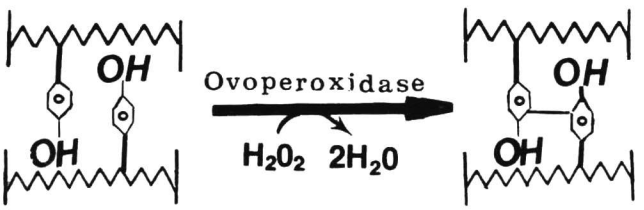
finely granular cores. Irwin and Threadgold (1972) suggested that this transformation in appearance may indicate some phase of the tanning process. According to Smyth and Clegg (1965), enzymatic oxidation of phenolic groups of tyrosine residues to quinone occurs without the removal of tyrosine from the protein chains. Irwin and Threadgold (1972) suggested that the rearrangement of protein molecules into a regular cross-linked shell could possibly explain its more electron-lucid appearance. The small areas with finely granular cores may represent sites of oxidation of phenolic groups of tyrosine to quinone, which polymerize to form melanin. Seed and Bennett (1980) suggested that, in Schistosoma mansoni, a lysine-rich protein or peptide may be critical in eggshell formation. Future studies which combine electron microscopy and autoradiography may further elucidate the relationship between these areas and quinone crosslinks in the eggshells of Acanthoparyphium spinulosum.

In contrast to egg capsules of A. spinulosum, those of Fasciola hepatica are colorless and transparent. According to Ramalingam (1973), the appearance of eggshells of F. hepatica is attributed to the

stabilization of the protein via formation of dityrosine crosslinks produced by the action of peroxidase. Seed and Bennett (1980) reported that dityrosine occurs in the eggshells of S. mansoni, but they suggested that the quantities of dityrosine are too small to account for hardening of protein in the eggshells. Foerder and Shapiro (1977) observed the occurrence of an ovoperoxidase-catalyzed mechanism for hardening of fertilization membranes of sea urchin eggs and documented the occurrence of such a mechanism in eggs of a wide variety of other taxonomic groups. A schematic representation of this mechanism is represented in Fig. 47.

This investigation also showed that a layer of reticular substance adhered to the inner and outer surfaces of the shell of Acanthoparyphium spinulosum. The concentric membranes associated with the reticular secretion indicate that the material may be similar to lipoproteins membranes that Clegg (1965) demonstrated on the inner and outer surfaces of the eggshells of Fasciola hepatica.

Finally, it was observed that a layer of electron-dense secretion adhered to the outer surface of eggshells in the distal uterus of A. spinulosum. This


OH
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secretion consisted of small clusters of round granules, resembling the secretory product of the dense body cells of the Mehlis' gland described by Burton (1967b). This observation is perhaps the first ultrastructural evidence of these secretory products in the distal uterus of trematodes. Detection of this material on the surface of encapsulated eggs in the uterus supports the theory that the dense body cells may serve to lubricate the capsule-filled uterus of trematodes (Henneguy, 1906; Kouri and Naus, 1038; Bogitsh, 1970; Irwin and Threadgold, 1972).

Based on the evidence obtained in this investigation, it is suggested that egg formation in A. spinulosum follows the general pattern of most other trematodes. However, a review of eggshell formation in other groups of animals, such as sea urchins, nematodes and amphibians, has raised a number of questions which concern the chemical nature and function of cortical granules in the ova of A. spinulosum.

It is possible that further research will generate data to ascertain whether there is a correlation between the release of cortical granules and the stabilization of eggshell membranes during fertilization in A.

spinulosum. Moreover, it will be of interest to determine whether dityrosine molecules occur in the eggshell of A. spinulosum and, if so, to what extent. It is also possible that peroxidase activity can be demonstrated in cortical granules in virgin oocytes of A. spinulosum.

CHAPTER VI

SUMMARY

1. Histochemical results from random sections of the reproductive system of Acanthoparyphium spinulosum showed that the components of the quinone-tanning system, i.e. basic proteins, phenols and phenolase were specifically localized in shell globules of vitelline cells and eggshells.
2. Ultrastructural studies of the ovary revealed that oocyte differentiation was characterized by organization of the endoplasmic reticulum and Golgi complex, which were involved in the synthesis of cortical granules. These granules were arranged immediately beneath the plasma membrane in mature oocytes.
3. Granular masses of ribosome-like particles, resembling "nucleolus-like bodies," occurred in the cytoplasm of maturing oocytes. The nucleus of mature oocytes appeared to have dispersed euchromatin. This observation may indicate the diplotene stage of prophase I.
4. In vitelline follicles, developing cells were characterized by orgazation of the granular

endoplasmic reticulum and Golgi complex, which were involved in the synthesis of shell globules. These globules were arranged immediately beneath the plasma membrane in mature cells. In contrast to immature cells, the cytoplasm of mature vitelline cells also contained lipid droplets and glycogen granules. Cytoplasmic extensions of nurse cells formed junctional complexes with maturing vitelline cells, suggesting that a mechanism of selective transport may exist between these cells.

5. The eggshell was initially deposited in the distal ootype and proximal uterus, which are penetrated by the membranous bodies of Mehlis' gland. Secretions from membranous bodies of this gland adhered to the inner and outer surfaces of the eggshells. The secretory product appeared as a layer of reticular substance associated with concentric membranes.
6. In contrast to young eggs, the shells of older ones were several times thicker and appeared to be blotched with electron-lucid areas, some of which contained finely granular cores. This transformation in appearance may indicate some phase of the tanning process.

7. Electron-dense secretions consisting of small clusters of round granules were seen to adhere to the outer surface of the egg capsules in the distal uterus. It is suggested that this material may represent the secretory product of dense body cells of Mehlis' gland.
8. Based on evidence in this investigation, it is concluded that egg formation in A. spinulosum follows the general pattern of that in most other trematodes.

LITERATURE CITED

Adamson, M. L. 1982. Ultrastructural observations on oogenesis and shell formation in Gyrinicola batrachiensis (Walton, 1929) (Nematoda: Oxyurida). Parasitology 86: 489-499.

Anderson, E. 1968. Oocyte differentiation in the sea urchin, Arbacia punctulata, with particular reference to the origin of the cortical granules and their participation in the cortical reaction. J. Cell Biol. 37: 514-539.

Andre, J. 1962. Contribution a la connaissance du chondriome. Etude de ses modifications ultrastructurales pendant la spermatogenese. J. Ultrastruct. Res., Suppl. 3: 1-185.

Balinsky, B. J. and R. J. Devis. 1963. Origin and differentiation of cytoplasmic structures in the oocytes of Xenopus laevis. Acta Embryol. Morph. Exp. 6: 55-108.

Beams, H. W. and R. G. Kessel. 1963. Electron microscopic studies on developing crayfish oocytes with special reference to the origin of yolk. J. Cell Biol. 18: 621-649.

_____. 1980. Ultrastructure and vitellogenesis in the oocyte of the crustacean, Oniscus asellus. J. Submicros. Cytol. 12: 17-27.

Bearup, A. J. 1960. Life history of Acanthoparyphium spinulosum Johnston, 1917 (Trematoda: Echinostomatidae). Austral. J. Zool. 8: 217-225.

Bhatnagar, A. K. and A. N. Gupta. 1984. Eggshell formation by vitelline globules or by uterus. An introspection. Acta Morphol. Neerl. Scand. 22: 75-83.

Bjorkman, N. and W. Thorsell. 1962. The fine morphology of the mitochondria from parenchymal cells in the liver fluke (Fasciola hepatica, L.). Exp. Cell Res. 27: 342-346.

_____. 1963. On the fine morphology of the formation of eggshell globules in the vitelline glands of the liver fluke (Fasciola hepatica, L.). Exp. Cell Res. 32: 153-156.

_____. 1964. On the ultrastructure of the ovary of the liver fluke (Fasciola hepatica, L.). Z. Zellforsch. Mikrosk. Anat. 63. 538-549.

Bogitsh, B. J. 1970. Observations on the cytochemistry

of the Mehlis' gland cells of Haematoloechus medioplexus. J. Parasitol. 56: 1084-1094.

Boyer, B. C. 1972. Ultrastructural studies of differentiation in the oocyte of the polyclad turbellarian, Prostheceraeus floridans. J. Morph. 136: 273-296.

Burton, P. R. 1960. Gametogenesis and fertilization in the frog lung fluke, Haematoloechus medioplexus Stafford (Trematoda: Plagiorchiidae). J. Morph. 107: 93-121.

_____. 1963. Histochemical study of vitelline cells, egg capsules and Mehlis' gland in the frog lung fluke, Haematoloechus medioplexus. J. Exp. Zool. 154: 247-258.

_____. 1967a. Fine structure of the reproductive system of a frog lung fluke. II. Penetration of the ovum by a spermatozoon. J. Parasitol. 53: 994-999.

_____. 1967b. Fine structure of the reproductive system of a frog lung fluke. I. Mehlis' gland and associated ducts. J. Parasitol. 53: 540-555.

Clegg, J. A. 1965. Secretion of lipoprotein by the Mehlis' gland in Fasciola hepatica. Ann. N. Y. Acad. Sci. 118: 969-986.

Clegg, J. A. and J. Morgan. 1966. The lipid composition of the lipoprotein membranes on the eggshell of Fasciola hepatica. Comp. Biochem. Physiol. 18: 573-588.

Clerot, J. C. 1968. Mise en evidence par cytochimie ultrastructurale de l'emission de proteines par le noyau d'auxocytes de Batraciens. J. Microscopie 7: 973-992.

Coil, W. H. 1965. Observations on eggshell formation in Hydrophitrema gigantica, Sandars, 1960 (Hemiuridae: Digenea). Z. Parasitenkd. 25: 510-517.

_____. 1966. Eggshell formation in the notocotylid trematode, Ogmocotyle indica (Bhalerao, 1942) Ruiz, 1946. Z. Parasitenkd. 27: 205-209.

Coil, W. H. and R. E. Kuntz. 1963. Observations on the histochemistry of Syncoelium spathulatum n. sp. Proc. Helminthol. Soc. Wash. 30: 60-65.

Dunn, M. C. 1959. Studies on the germ cell cycle of Neorenifer wardi (Byrd, 1936). Trans. Amer. Micros. Soc. 78: 385-408.

Erasmus, D. A. 1973. A comparative study of the reproductive system of mature, immature and "unisexual" female Schistosoma mansoni. Parasitology

67: 165-183.

_____. 1975a. Schistosoma mansoni: Development of the vitelline cell, its role in drug sequestration and changes induced by Astiban. Exp. Parasitol. 38: 240-256.

_____. 1975b. The subcellular localization of labelled tyrosine in the vitelline cells of Schistosoma mansoni. Z. Parasitenkd. 46: 75-81.

Foerder, C. A. and B. M. Shapiro. 1977. Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine crosslinks. Proc. Natl. Acad. Sci. USA 74: 4214-4218.

Frayha, G. J. and J. D. Smyth. 1983. Lipid metabolism in parasitic helminths. Adv. Parasitol. 22: 309-387.

Fried, B. and B. E. Stromberg. 1971. Eggshell precursors in trematodes. Proc. Helminthol. Soc. Wash. 38: 262-264.

Goldschmidt, R. 1908. Uber das verhalten des chromatins bei der eireifung und befrunchtung des Dicrocoelium lanceolatum Stil et Hass. Arch. Zellforsch. 1: 232-244.

Gonnert, R. 1962. Histologische untersuchungen uber

den feinbau der eibildungstatte (oogenotop) von Fasciola hepatica. Z. Parasitenkd. 21: 475-492.

Grant, W. C., R. Harkema and K. E. Muse. 1977. Ultrastructure of Pharyngostomoides procyonis Harkema 1942 (Diplostomatidae). II. The female reproductive system. J. Parasitol. 63: 1019-1030.

Gremigni, V. and L. Domenici. 1975. Genesis, composition and fate of cortical granules in eggs of Polycelis nigra (Turbellaria, Tricladida). J. Ultrastruct. Res. 50: 277-283.

Gremigni, V. and M. Nigro. 1983. An ultrastructural study of oogenesis in a marine triclad. Tissue and Cell 15: 405-415.

Gresson, R. A. R. 1958. The gametogenesis of the digenetic trematode, Sphaerostoma bramae (Muller) Luhe. Parasitology 48: 293-302.

_____. 1964a. Oogenesis in the hermaphroditic Digenea (Trematoda). Parasitology 54: 409-421.

_____. 1964b. Electron microscopy of the ovary of Fasciola hepatica, L. Quart. J. Micros. Sci. 105: 213.

Guilford, H. G. 1961. Gametogenesis, egg capsule formation and early miricidial development in the

digenetic trematode Halipegus ecentricus Thomas. J. Parasitol. 47: 757-764.

Gupta, V. and M. Puri. 1981. Eggshell formation in mammalian trematodes. Zool. Anz. 206: 252-256.

Halton, D. W., S. D. Strancock and A. Hardcastle. 1974. Vitelline development in monogenean parasites. Z. Parasitenkd. 45: 45-61.

_____.

1976. Fine structural observations on oocyte development in monogeneans. Parasitology 73: 13-23.

Hanumantha-Rao, K. 1959. Histochemistry of Mehlis' gland and eggshell formation in the liver fluke, Fasciola hepatica L. Experientia 15: 464-468.

_____. 1960. The problem of Mehlis' gland in helminths with special reference to Penetrocephalus ganapatii (Cestoda: Pseudophyllidea). Parasitology 50: 349-350.

Hathaway, R. P. 1979. The morphology of crystalline inclusions in primary oocytes of Aspidogaster conchiola von Baer, 1827 (Trematoda: Aspidobothria). Proc. Helminthol. Soc. Wash. 46: 201-206.

Henneguy, L. F. 1906. Recherches sur le mode de formation de l'oeuf ectolecithe du Distomum hepaticum. Arch. Anat. Micros. Morph. Exp. 9: 47-88.

Holland, N. D. 1979. Electron microscopic study of the cortical reaction of an ophiuroid echinoderm. Tissue and Cell 11: 445-455.

Hsu, W. S. 1962. An electron microscope study on the origin of yolk in the oocytes of the ascidian Boltenia villosa Stimpson. Cellule 62: 145-163.

Irwin, S. W. B. and J. C. Maguire. 1979. Ultrastructure of the vitelline follicles of Gorgoderina vitelliloba (Trematoda: Gorgoderidae). Inter. J. Parasitol. 9: 47-53.

Irwin S. W. B. and L. T. Threadgold. 1970. Electron microscope studies of Fasciola hepatica. VIII. The development of the vitelline cells. Exp. Parasitol. 28: 399-411.

_____. 1972. Electron microscope studies of Fasciola hepatica. X. Egg formation. Exp. Parasitol. 31: 321-331.

Johri, L. N. and J. D. Smyth. 1956. Histochemical approach to the study of helminth morphology.

Parasitology 46: 107-116.

Justine, J. L. and X. Mattei. 1984. Ultrastructural observations on the spermatozoon, oocyte and fertilization process in Gonapodasmius, a gonochoristic trematode (Trematoda: Digenea: Didymozoidae). Acta Zool. (Stockh.) 65: 171-177.

Kessel, R. G. 1966. An electron microscopic study of nucleolar cytoplasmic exchange in oocytes of Ciona intestinalis. J. Ultrastruct. Res. 15: 181-196.

_____. 1968. An electron microscopic study of differentiation and growth in oocytes of Ophioderma panamensis. J. Ultrastruct. Res. 22: 63-89.

Koulish, S. 1965. Ultrastructure of differentiating oocytes in the trematode Gorgoderina attenuata. I. The "nucleolus-like" cytoplasmic body and some lamellar membrane systems. Dev. Biol. 12: 248-268.

Koulish, S. and R. Kleinfeld. 1954. The cytochemistry of an inclusion body in the oocytes of Gorgoderina attenuata, a digenetic trematode. J. Histochem. Cytochem. 2: 481.

Kouri, P. and R. W. Naus. 1938. Formation of the eggshell in Fasciola hepatica as demonstrated by

histological methods. J. Parasitol. 24: 291-300.

Little, J. W., S. H. Hopkins and F. G. Schlicht. 1966. Acanthoparyphium spinulosum (Trematoda: Echinostomatidae) in oysters at Port Isabel, Texas. J. Parasitol. 52: 663.

Ma, L. 1963. Trace elements and polyphenol oxidase in Clonorchis sinensis. J. Parasitol. 49: 197-203.

Martin, W. E. and J. E. Adams. 1961. Life cycle of Acanthoparyphium spinulosum Johnston, 1917 (Echinostomatidae: Trematoda). J. Parasitol. 47: 777-782.

Massover, W. H. 1968. Cytoplasmic cylinders in bullfrog oocytes. J. Ultrastruct. Res. 22: 159-167.

McDowell, E. M. and B. F. Trump. 1976. Histologic fixatives suitable for diagnostic light and electron microscopy. Arch. Path. Lab. Med. 100: 405-414.

Mokhtar-Maamouri, F. 1980. Particularites des processus de la fecondation chez Acanthoparyphium filicolle Zschokke, 1888 (Cestoda, Tetraphyllidea, Onchobothriidae). Arch. Inst. Pasteur Tunis 57: 191-205.

Nellaiappan, K. and K. Ramalingam. 1980a. Specificity

of the enzyme phenol oxidase and possible metabolic pathway of sclerotization in Paraplerurus sauridae. J. Parasitol. 66: 217-219.

_____. 1980b. Prophenol oxidase and its activation in Paraplerurus sauridae (Digenea: Hemiuridae). Molec. Biochem. Parasitol. 2: 109-112.

Nollen, P. M. 1971. Digenetic trematodes: Quinone-tanning system in eggshells. Exp. Parasitol. 30: 64-72.

Nollen, P. M., A. L. Restaino and R. A. Alberico. 1973. Gorgoderina attenuata: Uptake and incorporation of tyrosine, thymidine and adenosine. Exp. Parasitol. 33: 468-476.

Nollen, P. M., R. D. Floyd, R. G. Kolzow and D. L. Deter. 1976. The timing of reproductive cell development and movement in Schistosoma mansoni, Schistosoma japonicum and Schistosoma haematobium, using techniques of autoradiography and transplantation. J. Parasitol. 62: 227-231.

Preston, C. M. and T. Jenkins. 1984. Trichuris muris: Structure and formation of the eggshell. Parasitology 89: 263-273.

Racker, D. K. 1983. Transmission Electron Microscopy: Methods of Application. Charles C. Thomas Publishers: Springfield, Illinois. 131 pp.

Ramalingam, K. 1970. Prophenolase and the role of Mehlis' gland in helminths. Experientia 26: 828.

_____. 1973. The chemical nature of the eggshell of helminths. I. Absence of quinone-tanning in the eggshell of the liver fluke Fasciola hepatica. Inter. J. Parasitol. 3: 67-75.

Rees, F. G. 1979. The morphology and the ultrastructure of the female reproductive ducts in the metacercaria and adult of Cryptocotyle lingula (Creplin) (Digenea: Heterophyidae). Z. Parasitenkd. 60: 157-176.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Robinson, R. D. and D. W. Halton. 1982. Fine structural observations on spermatogenesis in Corrigia vitta (Trematoda: Dicrocoeliidae). Z. Parasitenkd. 68: 53-72.

Sato, M., K. Sakoda, H. Nakashio and T. Noguchi. 1966. Electron microscopy of the eggshell formation in the

lung fluke, Paragonimus miyazakii. J. Electron Micros. 15: 286-287.

Seed, J. L. and J. L. Bennett. 1980. Schistosoma mansoni: Phenol oxidase's role in eggshell formation. Exp. Parasitol. 49: 430-441.

Seed, J. L., M. Boff and J. L. Bennett. 1978. Phenol oxidase activity: Induction by in vitro incubation. J. Parasitol. 64: 283-289.

Shropshire, P. A. 1984. The chemical nature of the eggshell membrane and other structures of the reproductive system in Acanthoparyphium spinulosum (Trematoda: Echinostomatidae) and Cloacitrema michiganensis (Trematoda: Philophthalmidae). M.S. Thesis. Atlanta University, Atlanta, Georgia.

Smyth, J. D. 1951. Eggshell formation in trematodes and cestodes as demonstrated by the methyl or malachite green techniques. Nature 168: 322-323.

_____. 1954. A technique for the histochemical demonstration of polyphenol oxidase and its application to eggshell formation in helminths and byssus formation in Mytilus. Quart. J. Micros. Sci. 95: 139-152.

Smyth, J. D. and J. A. Clegg. 1959. Eggshell formation

in trematodes and cestodes. Exp. Parasitol. 8: 286-323.

Spence, I. M. and M. H. Silk. 1971. Ultrastructural studies of the blood fluke Schistosoma mansoni. V. The female reproductive system - a preliminary report. S. Afr. J. Med. Sci. 36: 41-50.

Srivastava, M. and S. P. Gupta. 1976. Eggshell formation in Isoparorchis hypselobagri. Z. Parasitenkd. 49: 93-96.

_____. 1978. Polyphenol oxidase and scleroprotein/melanin pigments of Isoparorchis hypselobagri. Z. Parasitenkd. 55: 55-58.

Thangaraj, K., K. Nellaiappan and K. Ramalingam. 1982. Activation of prophenol oxidase in the liver fluke Fasciola gigantica Cobbold. Parasitology 85: 577-581.

Threadgold, L. T. 1982. Fasciola hepatica: Stereological analysis of vitelline cell development. Exp. Parasitol. 54: 352-365.

Threadgold, L. T. and S. W. B. Irwin. 1970. Electron microscopic studies of Fasciola hepatica. IX. The fine structure of Mehlis' gland. Z. Parasitenkd.

35: 16-30.

Thulin, J. 1982. Structure and function of the female reproductive ducts of the fish blood fluke Aporcotyle simplex Odhner, 1900 (Digenea: Sanguinicolidae). Sarsia 67: 227-248.

Wagner, U. M. and K. A. Seitz. 1984. Ultrahistology of oogenesis and vitellinogenesis in the spider mite Tetranychus urticae. Tissue and Cell 16: 179-194.

Willey, C. H. and S. Koulish. 1950. Development of germ cells in the adult stage of the digenetic trematode, Gorgoderina attenuata Stafford, 1902. J. Parasitol. 36: 67-79.

Yosufzai, H. K. 1953. Cytological studies on the oogenesis of Fasciola hepatica. La Cellule 55: 167-176.